Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 104 JULY 1960 No. 3

SECTION MEETINGS

CLEVELAND, O.	
Western Reserve University	May 16, 1960
ILLINOIS	
University of Illinois	May 10, 1960
IOWA	
State University of Iowa	April 19, 1960
SOUTHERN CALIFORNIA	
San Diego State College	May 14, 1960

Separation of Growth Promoting Activity from Horse Serum by Dialysis.* (25838)

D. P. METZGAR, JR.† AND M. MOSKOWITZ (Introduced by W. A. Hiestand)
Dept. of Biological Sciences, Purdue University, Lafayette, Ind.

Although a few tissue culture cell lines have been propagated serially in protein free medium(1), serum protein is required for serial propagation of most mammalian cell lines. It has been shown to play a role in the adhesion of cells to glass(2) and may also act as a carrier of as yet unidentified growth factors which are bound to protein and slowly released into the medium(3). In a study directed toward elucidation of some of the factors in serum required for cell growth it was observed that when serum was dialyzed against a synthetic medium or a salt solution under certain conditions, the dialysate supported growth of cells in cultures when it was used as a substitute for the normal serum supplement in synthetic medium. A method for separation of this fraction from serum and initial studies on its behavior will be presented.

Dialysis procedures. ¾ inch cellulose dialyzer tubing is inserted into a 6 oz. prescription bottle, the moderne oval model (E. H. Sargent Co.), so that about 1 inch of the open end overhangs the lip. Ten to 15 ml of double glass distilled water is placed in the bag and bottle is lightly capped with the plastic cap. The apparatus is sterilized by autoclaving at 121°C for 15 minutes. The water in the bag is replaced with serum and an equal amount of the dialyzing fluid is placed in the outer chamber. The excess dialyzer tubing is folded against the outside edge of the lip and the apparatus is tightly sealed with a rubber stopper. The apparatus is placed on its side in a 37°C incubator so that the sac comes in contact with the fluid.

* Aided by grants from Nat. Science Fdn. and Nat. Inst. Health.

† Predoctoral fellow of Nat. Inst. Health.

Test for protein. The dialysate was tested for protein contamination with 2.5% sulfosalicylic acid(4). The sulfosalicylic acid-dialysate mixture was allowed to stand for 2 hours at room temperature and was then examined in a narrow beam light source. This procedure was sensitive enough to detect protein corresponding to a 1:100,000 dilution of whole serum. *Tissue culture technic.* Cell strains used in these studies were the Mox strain (obtained from Dr. E. A. McCulloch, Univ Toronto), clone cultures derived from the Mox strain(5), HeLa. J-96, LLMC #1 (obtained from Dr. D. P. Gustafson, Purdue Univ), HEP 2 and Giardi human heart (obtained from Pitman Moore Co., Indianapolis, Ind.). These cells are normally maintained in a medium consisting of 20% horse serum and 80% synthetic medium CMRL 1066(6) with no added antibiotics. Dialysate medium was prepared by substituting undiluted dialysate for the serum portion of the medium. Five day cultures of cells grown in serum supplemented medium were washed in Hanks' basic salt solution(7) 3 times and suspended in dialysate medium so that final concentration was 50,000 cells per ml. One to 2 ml of this suspension was inoculated into tubes supplied with a 5% CO_2 atmosphere and incubated at 37°C. Total cell numbers were determined after 5 to 7 days incubation by counting in a hemocytometer and later, when it became available, in a Coulter electronic particle counter(8). Controls consisted of cells grown in serum supplemented medium and unsupplemented medium 1066. The Mox strain can be maintained in unsupplemented medium 1066 for a period of 10 to 15 days. During this time there is a slow, progressive degeneration of the cells with little evidence of multiplication. Periodic changes of the medium do not alter this picture and subcultures under these conditions have been unsuccessful.

Results. Initially, dialysis of horse serum was carried out at 4°C and 37°C for 144 hours against medium 1066, and the dialysate was tested for its ability to substitute for serum in supporting growth of cells. Increase in cell numbers in response to the 4°C and 37°C dialysates amounted to 2% and 81% respectively of total response of the cells to serum supplemented medium. The difference in activities of the dialysates suggested that the higher temperature may have caused release of growth promoting activity in the serum. Accordingly, whole serum was heated at 37°C for 7 days or longer, then dialyzed against medium 1066. A preparation was obtained under these conditions after 48 hours of dialysis which supported growth of the cells to the same extent as serum supplemented medium.

Sixty-four dialysates have thus far been prepared from 5 different lots of horse serum using this technic with either 1066 or Hanks basic salt solution as the dialyzing fluid, and all supported growth of cells (Table I). No detectable reaction was observed when these dialysates were tested with sulfosalicylic acid.

Thirteen serial passages of the Mox strain and 5 clone cultures derived from the Mox strain, and 8 passages of LLMC #1 have thus far been made in dialysate medium with no diminution in growth activity of the cells. Cells grown in dialysate medium are well flattened and possess extremely clear cytoplasm with smooth unbroken margins. They cling tenaciously to glass and are quite fragile. The 4 other cell strains: HeLa, J-96, HEP 2 and Giardi human heart grew in dialysate medium with the characteristic growth patterns observed in serum supplemented medium, but no quantitative determinations have as yet been made with these strains.

Discussion. All that can be said about the chemical nature of the growth-promoting activity is that it can pass through a cellulose dialyzer membrane, it does not react with sensitive protein precipitants and remains stable for several months at 37°C. Studies on its chemical nature are in progress.

Although the data in Table I clearly indicate that the dialysate is capable of supporting growth of cells to the same extent as serum, some of the dialysates were not as effective as serum in this respect, and individual

TABLE I. Growth of Mox Cells in Medium 1066 Supplemented with Serum, with Dialysate and Unsupplemented in 5 Serum Samples.

No. of dialysates prepared	Dialyzing fluid	Inoculum $\times 10^{-3}$	Cell numbers at 7 days $\times 10^{-3}$		
			Serum supplemented	Dialysate supplemented	Unsupplemented
6	1066	100	1222	991-728*	110
9	"	"	950	624-574	126
10	"	50	715	778-718	>10
6	Hanks	"	630	165-115	45
8	"	"	665	630-490	25
2	"	"	710	622-600	52
2	1066	"	710	670-587	52
2	Hanks	100	606	341-291	120
2	1066	"	606	560-470	120

* Maximum and minimum growth.

dialysates prepared from a given lot of serum varied in amount of growth response elicited. This variability may have been due to factors in the dialysis procedure which were not controlled or to the condition of the glassware at the time tests were carried out. During these experiments difficulties were encountered in washing of our glassware due to a change in the water suply. For example, when cells were placed in medium 1066 alone they would usually settle onto the glass and appear healthy for at least one week, but when this change in glassware was noted the cells were destroyed overnight. Cells grown in dialysate medium did not fare as well in this glassware as they did previously, but little or no effect was observed with cells in serum supplemented medium. In addition to promoting growth, serum probably contains factors that serve to detoxify glass surfaces. Serum dialysate apparently does not contain these factors and the condition of the glassware is thus more critical when cells are grown in dialysate supplemented medium than in serum supplemented medium.

Occasionally a dialysate was obtained which gave a reaction in the test for protein. This was probably due to some defect in the dialyzer membrane and these dialysates were discarded.

Summary. A method for separation of a fraction from horse serum which does not react in tests for protein and which is capable of substituting for whole serum in tissue culture medium has been described. When this fraction was used as a supplement in synthetic medium, cell growth was obtained which compared favorably with controls grown in serum supplemented medium.

1. Evans, V. J., Bryant, J. C., Fioramonti, M. C., McQuilken, W. T., Sanford, K. K., Earle, W. R., *Cancer Research,* 1956, v16, 77.

2. Fisher, H. W., Puck, T. T., Sato, G., *Proc. Nat. Acad. Sci., U. S.,* 1958, v44, 4.

3. Eagle, H., *Science,* 1959, v130, 432.

4. Layne, E., *Methods of Enzymology,* edited by S. P. Colowick and A. K. Kaplan, vIII, 1957.

5. Schenck, D., Moskowitz, M., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 30.

6. Parker, R. C., Castor, L. N., McCulloch, E. A., *Spec. Publ. N. Y. Acad. Sci.,* 1957, v5, 303.

7. Hanks, J. H., Wallace, R. E., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 196.

8. Richar, W. J., Breakell, E. S., *A. J. Clin. Path.,* 1959, v31, 384.

Received February 5, 1960. P.S.E.B.M., 1960, v104.

Antimicrobial Activity of Certain Marine Fauna. (25839)

C. P. Li
Division of Biologics Standards, Nat. Inst. of Health, Bethesda, Md.

The inhibitory effect of canned abalone juice on experimental poliomyelitis in mice and that of frozen fresh abalone juice on *Staphylococcus aureus in vitro* has been described(1). Following that discovery a number of sea animals were tested as possible source of antimicrobial agents. These included oysters (*Ostrea virginica*), clams, (*Venus mercenaris*), lobsters (*Homaridae americanus*), shrimps (*Crago vulgaris*), flounders (*Pseudo-pluronectes americanus*), butter fish (*Poronotus triacanthus*), and crab (*Callinectes sapidus*). *Abalone* (*Haliotis refescens*) was also included for positive control. The results are here reported.

Materials and methods. Fresh live oysters, clams, and lobsters; and fresh shrimps, flounders and butter fish kept on ice were purchased in the sea food market. Cooked crab meat was purchased in the food market. Abalone was obtained from California in frozen state.* A penicillin sensitive strain, FDA 209, and a penicillin resistant strain of *Staphylococcus aureus* and a strain of *Bacillus subtilis* were used as test organisms.† *Preparation of extract.* Oysters, clams and tails of lobsters without shells, shrimp without heads and shells, flounders and butter fish without viscera and white crab meat were ground separately in meat grinder. Ground tissue was heated in container with equal weight of water at 90-95°C for 30-45 minutes. Heated material was centrifuged at 1,500 rpm for 15 minutes and the supernatant fluid was kept at –20°C before assay. Viscera with their contents, of abalone, lobsters, flounders and butterfish were also prepared as described above. The viscous grayish-white fluid left in container for oysters was regarded as oyster juice. A brownish fluid obtained from frozen fresh whole abalone upon thawing was regarded as abalone juice. These juices were heated to 70°C for 30 minutes and centrifuged at 1,500 rpm for 15 minutes before assay. No extract of abalone meat was tested in the present study. The pH of all extracts or juice was adjusted between 6.9 and 7.1 before assay. *Method of assay.* This was intended only for screening accuracy. Three consecutive 24-hour transfers of *Staphylococcus aureus,* FDA 209 strain, were made prior to using the culture as inoculum. Difco nutrient medium at pH 7.0 was used for all transfers and for assay. Ten-fold dilutions of 24-hour culture were made in buffered saline and a loopful (5 mm in diameter) of each dilution was inoculated into a Wassermann tube containing 1.8 ml of nutrient broth with 0.2 ml of the extract or juice to be tested. Corresponding dilutions of the same test organisms were also inoculated into a series of tubes with 2.0 ml of broth alone. After incubating at 35°C for 18 hours, a loopful from each tube was spread on a quarter of a nutrient agar plate which was then incubated at 35°C overnight and colonies of growth thereon were counted. Absorbent paper disc method for assay of antibiotics was occasionally used. Paper discs impregnated with test material were placed on surface of agar plates upon which a 24-hour broth culture had previously been spread. After overnight incubation a clear zone around the disc indicated inhibition.

Results. Abalone juice, oyster juice and extract and clam extract all demonstrated antimicrobial activity while all others were apparently negative as revealed by fluid method of assay (Table I). This method was used instead of conventional absorbent paper disc method, because the latter gave only negative or inconsistent positive results for abalone juice, as mentioned previously(1). However, in the present study when the penicillin sensitive strain of *Staphylococcus aureus* was used as a test organism, clam extract showed rather

* This was kindly supplied by Dr. H. G. Orcutt, Bureau of Marine Fisheries, Calif.

† These cultures were kindly supplied by Dr. A. L. Schade of Nat. Inst. of Allergy and Infect. Diseases.

TABLE I. Effect of Juice or Extract of Various Sea Animals on Growth of *Staphylococcus aureus*, FDA 209 Strain.

Juice or extract of sea animals		Colonial growth from 1 loopful of 18 hr broth cultures inoculated with serial dilutions							
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	Control
Abalone juice	+	0	0	0	0	0	0	0	0
	—	Con	Con	Con	Con	0	0	0	0
Oyster "	+	9	4	0	0	0	0	0	0
	—	Con	Con	Con	Con	TN	0	0	0
Oyster extract	+	105	0	0	0	0	0	0	0
	—	Con	Con	Con	Con	Con	0	0	0
Clam "	+	0	0	0	0	0	0	0	0
	—	Con	Con	Con	Con	Con	0	0	0
Lobster "	+	Con	Con	Con	TN	TN	TN	0	0
	—	"	"	"	Con	Con	0	0	0

Control: Broth with test material but not inoculated.
9, 4, etc.: Indicating No. of colonies.
TN: Colonies too numerous to be counted but not confluent.
Con: Confluent growth.
+ = present; — = absent.
Results of crab, flounder, shrimp and butter fish are similar to that of lobster.

consistently a 3 mm inhibiting zone around the disc while oyster extract was negative. By the fluid method of assay, clam extract also demonstrated moderate inhibitive action against a penicillin resistant strain of *Staphylococcus aureus*.

Extracts of viscera of some of these sea animals did not show definite or marked inhibitory effect. However, on agar plates inoculated with a broth culture containing viscera extract of abalone, colonies of *Staphylococcus aureus* were not only much less numerous but also much smaller in size than those in the controls, indicating some inhibition. In separate studies‡ now in progress, it is shown that, after chemical treatment, abalone juice and abalone viscera extract both possess marked antimicrobial activity against penicillin sensitive and penicillin resistant *Staphylococcus aureus* and *Bacillus subtilis* in a concentration as low as 1.0 mg %.

Discussion. These experiments extend our previous study on antimicrobial activity of abalone juice. The fact that an inhibitory effect was demonstrated much more easily in fluid medium than on solid agar suggested that the active agent was not readily diffusible in the latter medium. The active agent was heat stable, and non-dialyzable(1). Its properties are not typical of any known antibiotics(2,3) which are usually readily diffusible on solid agar medium, heat labile and dialyzable. It was not determined whether the action was bactericidal or bacteriostatic.

Whether the agent was located in viscera contents or in body tissue, and whether there were more than one agent cannot now be stated. It is possible that some antimicrobial agent or agents present in the aquatic environment are ingested by sea animals, undergo chemical changes *in vivo,* and result in a modified but active antimicrobial compound or compounds. The antimicrobial effect of some of the agents concerned can only be demonstrated when they are freed from accompanying nutritious substances and growth factors. Further work along such lines including *in vivo* experiments and virus studies is in progress.

Summary. A limited number of sea animals were screened for antimicrobial activity against *Staphylococcus aureus*. In addition to abalone juice, whose antimicrobial activity had been reported, extracts of oysters and clams possessed marked inhibitive effect. Substance of this nature may be valuable therapeutic and prophylactic tools.

The author wishes to thank Dr. C. W. Hiatt for his enthusiastic suggestions, J. L. Rogers whose energetic efforts carried the load of technical pro-

‡ Chemical purification is being carried out by Dr. B. Prescott, Nat. Inst. of Allergy and Infect. Diseases.

cedure and W. G. Jahnes, E. C. Martino and E. W. Harvey for technical assistance.

1. Li, C. P., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 522.

2. Pratt, R., Dufrency, J., *Antibiotics,* J. B. Lippincott Co., Philadelphia, 1949.

3. Goldberg, H. S., *Antibiotics: Their Chemistry and Non-medical Uses,* D. Van Nostrand Co., 1959.

Received February 3, 1960. P.S.E.B.M., 1960, v104.

Time Study of Acute Cold-Induced Acceleration of Thyroidal I^{131} Release in the Hamster.* (25840)

KARL M. KNIGGE

Dept. of Anatomy, School of Medicine, University of Cincinnati, Ohio

Previous experiments(1,2) have shown that rate of hormone release from hamster thyroid is accelerated markedly during first 12 hours exposure to cold (5-6°C). The data indicated that this acute release of thyroidal I^{131} is dependent upon a neural mechanism which discharges TSH from the pituitary gland. The present study is a more careful analysis of some hypothalamo-pituitary-thyroid relationships during this initial 12 hour period of cold.

Methods. Adult hamsters of both sexes weighing 90-110 g were used. Twelve to 18 hours after intraperitoneal injection of 4-8 μc of I^{131}, thyroidal uptake was measured by counting externally neck and hind-leg regions with shielded scintillation crystal. The neck-minus leg counts, expressed as per cent of injected dose, represented thyroid uptake. The fractional rate of thyroidal I^{131} release was estimated by subsequent daily measurement of thyroid activity, each day's calculation being corrected for physical decay and expressed as percentage of initial 12 hour uptake. After at least 3 days of external counting to determine normal slope of release curve and immediately after zero count at room temperature (20-22°C), groups of hamsters were exposed to cold (5-6°) for following periods: 0.5, 1, 2, 3, 4, 6, 8 and 12 hours. During periods of cold exposure, animals were housed individually in metal cages with minimum of bedding; free access to food and water was permitted. After desired period of cold, thyroid activity was again measured externally, blood samples taken for determination of PBI^{127} and thyroid glands removed and weighed. Radioactivity was measured on one excised lobe, then prepared for determination of total iodine content. Posterior lobes of pituitary glands were separated by blunt dissection and adenohypophyses of at least 8 animals of each group were pooled, homogenized in acidified saline and frozen until assayed for thyrotropin (TSH) content. A time-study of response of hamster thyroid to exogenously administered TSH was carried out in same fashion as the time-study of thyroid response to cold. After zero external count, not less than 10 animals/group were injected intraperitoneally with one unit of TSH (Armour Thtropar). Ability of thyroxine to block cold-induced release of thyroidal I^{131} was examined. Freshly dissolved L-thyroxine was administered subcutaneously to groups of not less than 8 animals in doses ranging from 2.5 to 20 μg at varying times before and after beginning of 12 hours of cold exposure.

Results. Response to cold. To express quantitatively the accelerated release produced by varying periods of cold exposure, normal (room temperature) release curves of each animal were continued, appropriate number of hours (corresponding to number of hours of cold) to determine a theoretical position of the curve at room temperature. The difference between this point and the actual position of release curve determined after cold exposure is considered per cent of accelerated release. Fig. 1 summarizes time sequence of accelerated release from hamster thyroid at-

* Supported by U.S.P.H.S. and N.S.F. grants.

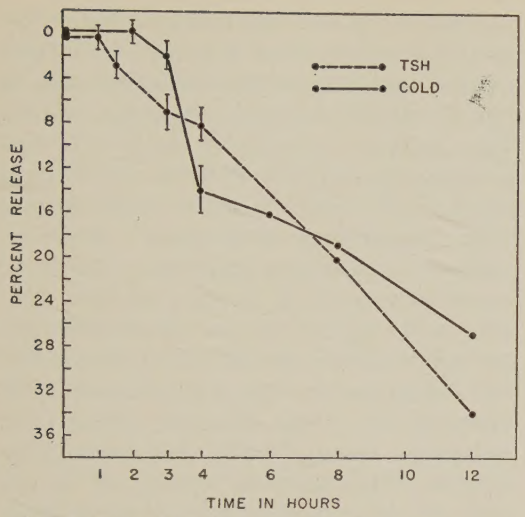


FIG. 1. Thyroidal I^{131} release in the hamster following cold exposure or thyrotropin administration.

tendant upon acute cold exposure. No accelerated release occurs during first 2 hours of cold; after 3 hours a detectable (but not statistically significant) change in rate of release is seen. During 3rd to 4th hour of cold a maximal rate of release occurs. After 4th hour, the rate is slower but continues to fall until a 25-30% release is achieved at 12 hours of cold. Cold-induced disappearance of thyroidal I^{131} from the gland is paralleled by a fall in chemically determined iodine content (Table I). A discrepancy is noted, however, in that after 2 hours of cold no evidence of release based upon thyroidal I^{131} measurements is seen, while total iodine determinations indicate a fall of approximately 18%. Although the discrepancy between these 2 measurements of thyroid response cannot be fully explained, it may represent intraglandular deiodination(3) or a pool of thyroidal organic and inorganic iodide(4) which is not radio-labeled and which responds earlier to administration of TSH. During the critical period of first 4 hours of cold exposure PBI, values were determined on samples of blood from each of 6 control animals and 6 exposed for 2, 3 and 4 hours. PBI level in control hamsters is 2.81 $\pm$ .31 mg %. No significant change in PBI level occurs during 2-4 hours of cold exposure, the respective values being 2.83, 2.78 and 2.83 mg % (Table I).

Response to Thyrotropin. A significant acceleration in thyroidal I^{131} release produced by exogenously administered TSH is first observed 1.5 hours after administration. Rate of accelerated release continues thereafter at approximately the same slope throughout the 12 hour period studied and produces a slightly greater final total per cent release than does 12 hours of cold exposure (Fig. 1).

Pituitary Thyrotropin. TSH content in hamster pituitary was estimated by using the assay of Greenspan *et al.*(5), based upon uptake of P^{32} by chick thyroid. USP thyrotropin standards were used in doses of 0.4 and 4.0 milliunits. Extracts of pooled pituitary glands from several groups of animals were adjusted to administer 0.1 ml intracardially at 2 dose levels estimated to be equal to that of the standards. Seven chicks were used/group with 5 μc P^{32} being administered subcutaneously 3 hours after TSH and the chicks killed 3 hours after radiophosphorus. Thyroids were dissected under a binocular dissecting microscope, dried and their radioactivity measured using a thin window GM tube. Pituitary TSH content of cold-exposed hamsters and corresponding control (room temperature) animals was determined in 3 separate assays for each cold period studied. TSH content after 2 hours of cold was determined in 2 assays.

No significant sex difference in TSH concentration is found between pituitary glands of male and female hamsters, normal concentration being 0.68 $\pm$ .11 milliunit/mg (Table II). A 60% decrease in TSH concentration results after one hour. This decreases further until a concentration of 0.16 milli-

TABLE I. Thyroid Iodine Concentration and PBI after Acute Cold Exposure of the Hamster.

Hr after cold	Thyroid I^{127}, μg/100 mg	PBI, μg %
0	(16) 15.1 $\pm$ 1.1*	(6) 2.81 $\pm$ .31
2	(12) 12.4 $\pm$ .9	(6) 2.83 $\pm$ .22
3		(6) 2.78 $\pm$ .30
4	(8) 11.3 $\pm$ 1.4	(6) 2.83 $\pm$ .18
6	(8) 10.6 $\pm$ 1.3	
8	(8) 10.1 $\pm$ .8	
12	(9) 9.7 $\pm$ .8	

* No. of determinations (in parentheses), mean $\pm$ S.D.

TABLE II. Influence of Cold Exposure upon Concentration of Thyrotropin in Pituitary Glands of Hamsters.

Cold exposure, hr	No. animals	No. assays	Pituitary wt, mg	Pituitary TSH, mu/mg
0	38	6	3.08 ±.04†	.68 ±.11
0.5	18	3	3.44 ±.07	.54 ±.13
1	16	3	3.26 ±.03	.24 ±.06*
2	13	2	3.46 ±.07	.38 ±.08*
4	18	3	3.21 ±.07	.30 ±.10*
7-8	18	3	3.38 ±.10	.17 ±.05*
12	18	3	3.58 ±.06	.16 ±.07*

* Significant difference (P = .001) from control.
† Mean ± S.D.

unit/mg is present at 7-12 hours after cold.

Inhibition of release by thyroxine. Inhibition of cold-induced acceleration was considered complete when thyroidal I^{131} release of less than 10% was observed after 12 hours at 5-6°C. A release of greater than 25% represented no inhibiting effect. Single doses of 2.5 and 5 μg of thyroxine inhibit thyroidal I^{131} release in hamsters maintained at room temperature, but are unable, regardless of when administered, to inhibit accelerated release precipitated by 12 hours of cold exposure. From 2 hours before until 2 hours after cold exposure, 10 μg of thyroxine blocks release consistently. After 4 hours of cold, 10 μg of thyroxine is ineffective while at 3 hours, results are inconsistent with approximately equal numbers of animals exhibiting no block, partial, or complete inhibition of release.

Discussion. Influence of the central nervous system in normal pituitary-thyroid relationships is demonstrated by evidence that electrical destruction of various hypothalamic regions results in disability of pituitary gland in supplying increased amounts of thyrotropin under a variety of experimental conditions (goitrogens, cold exposure, thyroidectomy) which normally evoke pituitary-thyroid responses(6-9). Under proper conditions, acute cold appears to evoke a neural mechanism which discharges pituitary TSH similar to stress-induced release of ACTH. Our experiments permit a more detailed description of neural and hormonal events occurring during initial 12 hours of cold exposure of the hamster. Time-study of thyroidal I^{131} release in response to cold reveals that acceleration begins at or shortly after 3 hours. TSH (exogenous) produces a detectable increase in rate of release 1.5 hours after administration. Time sequence of action of one dose of exogenously administered TSH cannot be compared strictly with the action of endogenous TSH released from the pituitary. However, sudden depletion of a major part (60%) of the pituitary store of TSH during 0.5-1 hour after cold, appears almost comparable to a one-injection phenomenon itself. This is further emphasized by very rapid acceleration in thyroidal I^{131} release observed during third and fourth hours of cold. Intraperitoneally injected TSH, absorbed rapidly but apparently at a more constant rate, produces accelerated release whose slope is more constant throughout the 12 hours studied. Acute cold exposure of rat(10) and rabbit(11) also evoke neural mechanisms for discharge of TSH. In rabbits, increased blood TSH is detectable within 30 minutes of exposure; maximal amounts (500-fold increase) are achieved after 3 hours(12). PBI determinations revealed that no major change in peripheral levels of thyroid hormone were responsible for initiation of TSH discharge and thyroidal I^{131} release. It is possible that pituitary and/or responsive CNS areas are more sensitive to circulating levels of thyroxine than is revealed by this parameter.

The blocking effect of thyroxine bears significantly upon the question of site of action of thyroxine in feed-back control mechanisms operating between thyroid and pituitary. Thyroxine blocked thyroidal release when administered 2-3 hours after cold. Once accelerated, release had begun from the thyroid (at or shortly after the third hour) thyroxine had no effect. Additionally, the fact that the major stimulating surge of pituitary TSH was released within one hour after cold and exogenous thyroxine was then still able to inhibit thyroidal release when administered at 2-3 hours after cold, leads to the possibility that under these conditions of cold exposure of the hamster, thyroxine may be acting at thyroid level to prevent thyroidal release or in some way interfere with the action of TSH

in evoking release. A feed-back effect at pituitary level has been demonstrated by direct micro-injection of thyroxine into the pituitary (13,14). Yamada and Greer(14) and Yamada(15) have, furthermore, demonstrated an inhibitory effect on TSH release by micro-injection of thyroxine into anterior hypothalamus of the rat. Integrity of these hypothalamic areas is, however, not essential for feed-back inhibition since thyroxine is still effective after electrolytic destruction of anterior hypothalamus(16,17). A possible role of other brain areas is suggested by Mess(18-20) who demonstrated that the depressing effect of thyroxine upon thyroid weight and pituitary TSH content was notably modified by lesions of the habenula complex.

Summary. Analysis of time sequence in pituitary and thyroid phenomena involved during acute response of hamsters to cold exposure indicates a rapidly activated neural component. Within 0.5-1 hour after cold, sensory perception of this temperature change has generated sufficient input into hypothalamic effector mechanisms to deplete pituitary TSH by 60%. After a latent period of 1.5 hours, accelerated release begins and results in 25-30% decrease of thyroidal I^{131} during 12 hours of cold. Thyroxine (10 μg) is capable of inhibiting thyroidal I^{131} release when administered as long as 2-3 hours after exposure to cold.

1. Knigge, K. M., Goodman, R. S., Solomon, D. H., *Am. J. Physiol.,* 1957, v189, 415.

2. Knigge, K. M., Bierman, S. M., *ibid.,* 1958, v112, 625.

3. Rosenberg, I. N., Athans, J. C., Behar, A., *Endocrinology,* 1960, v66, 185.

4. Nagataki, S., Schizume, K., Matsuda, K., Ishii, J., PROC. SOC. EXP. BIOL. AND MED., 1959, v102, 765.

5. Greenspan, F. S., Kriss, J. P., Moses, L. E., Lew, W., *Endocrinology,* 1956, v58, 767.

6. Ganong, W. F., Frederickson, D. S., Hume, D. M., *ibid.,* 1955, v57, 355.

7. Bogdanove, E. M., *ibid.,* 1957, v60, 689.

8. Reichlin, S., *ibid.,* 1957, v60, 567.

9. Florsheim, W. H., *ibid.,* 1958, v62, 783.

10. Brown-Grant, K., *J. Physiol.,* 1956, v131, 52.

11. Brown-Grant, K., von Euler, C., Harris, G. W., Reichlin, S., *ibid.,* 1954, v126, 1.

12. Bottari, P., *Ciba Colloquia on Endocrinology,* 1957, v11, 52.

13. von Euler, C., Homgren, B., *J. Physiol.,* 1956, v131, 125.

14. Yamada, T., Greer, M. A., *Endocrinology,* 1959, v64, 559.

15. Yamada, T., *ibid.,* 1959, v65, 216.

16. D'Angelo, S. A., *J. Endocrinology,* 1958, v17, 286.

17. Florsheim, W. H., PROC. SOC. EXP. BIOL. AND MED., 1959, v100, 73.

18. Mess, B., *Endokrinologie,* 1958, v35, 196.

19. ———, *ibid.,* 1958, v35, 296.

20. ———, *ibid.,* 1959, v37, 104.

Received March 7, 1960. P.S.E.B.M., 1960, v104.

Cholesterol Concentration in Human Serum and Blood Cells.* (25841)

MARIO MANCINI† AND ANCEL KEYS

Lab. of Physiological Hygiene, University of Minnesota and Hastings State Hospital, Hastings

The concentration of cholesterol in human erythrocytes has been reported to be much less variable than in plasma and relatively independent of the latter value(1,2). However, the data of Foldes and Murphy(3) from 20 "normals" aged 19 to 35 and 20 surgical patients aged 70 to 90 indicate considerable variation between individuals, the standard deviation of red cell concentration in the normals being 27.6 mg per 100 ml, or 16% of the mean of 173.0. The variability was equally large when the 2 age groups were separately considered. Moreover, when plasma cholesterol changes were induced by treatment of 17 thyroid patients the data reported indicate cell cholesterol concentration changes in the

* The work was aided by research grants from U.S.P.H.S. and Minn. Heart Assn.

† Present address: Istit. di Patologia Speciale Med., Univ. di Napoli, Italy.

same direction as those in the plasma(4). Cell concentration in these studies was calculated from measurements of the hematocrit and concentrations in the whole blood and in the plasma. The present paper reports the findings from both indirect and direct methods of estimating cholesterol in bloods from a sample of men and from men in dietary experiments which induced large changes in the serum cholesterol level.

Methods and procedures. Casual venous blood samples were drawn from 38 men, mostly ostensibly healthy but including some patients with coronary heart disease. Ages ranged from 28 to 65 years, the mean being 57.4 years. Six schizophrenic men, aged 40 to 57 years, long resident at the Hastings State Hospital, were studied in connection with controlled dietary alterations. After the men were stabilized on a diet fixed as to kind and amount of fat, the diet was changed by substituting, isocalorically, carbohydrates and poly-unsaturated fats for most of the saturated fats or vice versa. Periods of 3 weeks on each diet were used and blood samples were drawn on the last 2 days on each diet. Plasma and whole blood cholesterol was estimated in duplicate 0.1 ml samples by the method of Abell, *et al.*(5) as modified by us (6). The method was checked against the more specific method involving precipitation as the digitonide and no systematic difference was found. Cell cholesterol concentration was estimated directly by the same method on 0.2 ml aliquots, in duplicate, of a one-to-one dilution of washed cells with distilled water. The hematocrit value was obtained by centrifuging for 60 minutes at 1700 rpm (800 x g) in Wintrobe tubes. In all cases precautions were taken to prevent hemolysis and the serum was separated immediately after clotting.

Results. The standard error of 50 pairs of duplicate direct analyses of cell cholesterol was $\pm$ 2.4 mg/100 ml, calculated as $(S.E.M.)^2 = (\Sigma\Delta^2)/2N$, when N is number of pairs and Δ is difference between duplicates. This is of the same order as, but actually slightly smaller than, standard error of measurement found with the same method applied to serum(7).

Results on 38 casual blood samples are summarized in Table I. Indirect and direct estimates of cell cholesterol concentration gave excellent agreement between the means 137.6 and 137.7 mg/100 ml respectively. The variability of the value obtained indirectly was almost twice that of the direct estimate but even so was only a small fraction of the serum variability. Concentration of cholesterol in the cells was therefore relatively constant and was not correlated ($r = 0.07$) with serum concentration.

TABLE I. Total Cholesterol Concentration in mg/100 ml in Serum and in Cells in Blood Samples from 38 Men.

	Serum	Cells	
		Direct	Indirect
Mean	238.8	137.7	137.6
Stand. dev. (inter-individual)	48.8	6.5	10.5

Fig. 1 summarizes results from the dietary experiments. In the face of a mean serum change of –63.8, corresponding cell values, measured directly, showed a mean change of only –3.6 mg/100 ml. With a serum change of +51.8, the cell change averaged –2.0 mg/100 ml. These cell concentration changes are statistically not significant.

Discussion. The values for cell cholesterol reported by Foldes and Murphy(3,4) are higher and far more variable than our findings. The Bloor method they used gives values for plasma or serum averaging about 15% higher than the present method. This is explained by the fact that cholesterol esters give a more intense color with the Liebermann-Burchard reagent than does free cholesterol. Since pure free cholesterol is always used as the standard it is obvious that a method that does not hydrolyze the esters

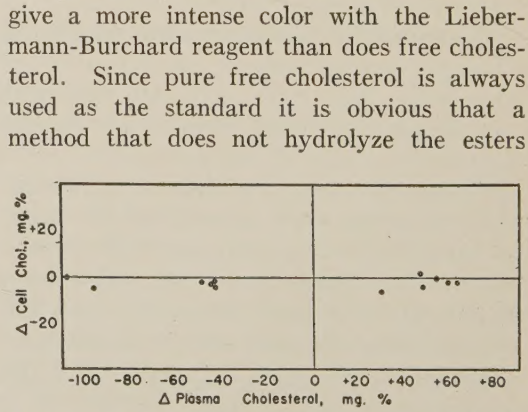


FIG. 1. Changes in plasma and in erythrocyte cholesterol concentration induced by dietary alterations. Each point represents change in blood concentration of an individual between 2 diets.

must give erroneously high values. But on detailed computation this factor does not seem to account fully for the discrepancy between our mean values and those of Foldes and Murphy though it could account for some of the greater variability of the latter data.

It is well known that all or almost all of the cholesterol in erythrocytes is in the free form(1,2,8) whereas except in a few disease states the ester form is a constant fraction of about 71% of total cholesterol in plasma or serum. There is a rapid exchange of labelled cholesterol between cells and plasma(9,10) but apparently the cells offer only a fixed number of acceptor sites for free cholesterol and admit no ester cholesterol. Since when cells are laked the cholesterol in them all appears in the stroma(11), it follows that the cholesterol acceptor sites in the cells are in that portion. It seems reasonable to suggest that any inter-individual variability in cell cholesterol concentration must be related to individual differences in the relative volume or surface of the stroma per unit volume of the erythrocytes. All of the present data suggest that the stroma per unit volume of erythrocytes must normally be very constant although a very small real variability between individuals is indicated.

Summary. 1) Direct analyses of cholesterol in blood cells from 38 men yielded average value of 137.7 mg/100 ml and there was no correlation with serum values in these samples. Standard deviation of these cell values was only 6.5 and part of this small value is accounted for by analytical error. Indirect estimates agreed closely but variability was larger. 2) In 2 dietary experiments on 6 men which produced average changes of −63.8 and +51.8 mg of cholesterol/100 ml of serum, there were no significant changes in cholesterol concentration in the cells.

We acknowledge the help of Dr. Joseph T. Anderson in chemical analyses and of Jaakko Kihlberg in the statistical work.

1. Boyd, E. M., *Canad. J. Res.*, 1937, vD15, 1.
2. Rubin, S. H., *J. Biol. Chem.*, 1939, v131, 691.
3. Foldes, F. F., Murphy, A. J., Proc. Soc. Exp. Biol. and Med., 1946, v62, 215.
4. ———, *ibid.*, 1946, v62, 218.
5. Abell, L. L., Levy, B. B., Brodie, B. B., Kendall, F. E., *J. Biol. Chem.*, 1952, v195, 357.
6. Anderson, J. T., Keys, A., *Clin. Chem.*, 1956, v2, 145.
7. Keys, A., Anderson, J. T., Grande, F., *Lancet* (II), Nov. 16, 1957, 959.
8. Sperry, W. M., *J. Biol. Chem.*, 1935, v111, 467.
9. Hagerman, I. J., Gould, R. G., Proc. Soc. Exp. Biol. and Med., 1951, v78, 329.
10. London, I. M., Schwartz, H., *J. Clin. Invest.*, 1953, v32, 1248.
11. Erickson, B. N., *J. Biol. Chem.*, 1938, v122, 507.

Received March 11, 1960. P.S.E.B.M., 1960, v104.

Selective Inhibitory and Teratogenic Effects of 2,5-Alkylbenzimidazole Homologues on Chick Embryonic Development.*† (25842)

Unabelle Boggs Blackwood‡ (Introduced by M. S. Shorb)
University of Maryland, College Park

Many studies have shown that compounds differing widely in molecular structure, as well as more closely related compounds, elicit specific teratogenic responses(1-7). In addition, compounds as closely related as homologues also have this ability as demonstrated by Ambellan(8) with phosphonucleotides in amphibians. Homologous alkyl benzimidazoles had a characteristic order of inhibition. Hendlin and Soars(9) first reported that increasing length of alkyl chain in the 2-position from 0 to 4 carbons caused a corresponding increase in inhibitory activity in bacteria. Similar results were found in chicken sperm with alkyl chains of 1 to 7 carbons(10). However, Tamm *et al.*(11) found that lengthening the chain from 2 to 4 carbons did not cause further increase in inhibition of virus multi-

plication; similarly, Blackwood and Shorb (12) found that the 7-carbon chain was no more lethal in the early embryo than the 2-carbon chain. Our purpose is to report selective teratogenic effects and inhibitory patterns of 2,5-dimethylbenzimidazole (DMB), 2-ethyl-5-methylbenzimidazole (EMB), and 2-hepta-5-methylbenzimidazole (HMB) when administered to chick embryos prior to incubation.

Materials and methods. The eggs were from 2 strains of White Leghorn hens (Mt. Hope and a random bred strain) mated to males of either Mt. Hope, random bred or flightless strains. Benzimidazoles previously mentioned were dissolved in dilute HCl and injected into the albumen through the air cell. Eggs were candled 5th day of incubation and daily thereafter to 18th day. Each infertile and dead embryonated egg was removed and immediately opened for examination. Dead or severely malformed living embryos were examined under stereoscopic microscope and staged according to Hamburger and Hamilton (13). All data represent arithmetic average of 2 to 5 repeats with approximately 15 fertile eggs/repeat. Control groups, which included non-treated eggs and eggs injected with dilute HCl (.06-0.1 N) or Vit. B_{12} dissolved in water, consistently displayed a low mortality, about 5% (average of 14 repeats, 286 eggs; no repeat exceeded 11%). Further details of storage and incubation, method of dissolving compounds, injection procedure and control groups have been previously described and discussed(12,14). Statistical analysis was not made because of the involved nature of the data. Since variation was inevitable, trends and correlation of different indicators of embryonic response are relied upon rather than absolute quantities. Embryonic response was determined by mortality, incidence of embryos ceasing development at successive periods throughout incubation, and types and incidence of types of malformations.

Results. A. *Inhibition patterns.* Plotting graphically the stages of development of dead or severely malformed early living embryos against cumulative per cent dead, curves of inhibition were obtained which followed a pattern characteristic of each compound. These graphs give 3 different types of information other than the general picture of the total curve: a) total mortality resulting from each treatment, b) incidence of embryos which ceased development at each stage period, and c) total per cent (thereby proportion) of embryos ceasing development at end of any period. Total mortality data were based on embryos dead through 18 days; however, development did not appear to proceed beyond approximately 14 days in those embryos dying between 14 and 18 days. When equivalent doses (0.50 mg DMB and EMB, 0.70 mg HMB which is approximately 3 μM) of the 3 compounds were thus compared (Fig. 1), EMB and HMB were similarly lethal but caused greater mortality than DMB. However, inhibition curves of EMB and HMB differed considerably while those of EMB and DMB were similar. For example, EMB caused a large proportion of embryos to cease development before stage 17 while HMB failed to stop development during this period.

Increasing doses of DMB did not increase mortality, and predominately inhibited development at 2 periods (stages up to 19 and 32-40) (Fig. 2). Contrary to this effect, dose increase of EMB (Fig. 3) caused a corre-

* Scientific Article No. A787. Contribution No. 3052 of Maryland Agr. Exp. Station (Dept. of Poultry Husbandry). Supported by grants from Nat. Inst. of Arthritis and Metab. Dis. Material was taken in part from dissertation submitted to Univ. of Md. in partial fulfillment of requirements for Ph.D. degree.

† The author is grateful to Dr. Mary S. Shorb under whose supervision most of the work was done, also for encouragement and helpful advice; to Drs. C. S. Shaffner and R. M. Johnson for assistance in editing the manuscript; to Drs. D. Hendlin and K. Folkers, Research Laboratories, Merck Sharp & Dohme, Inc., Rahway, N. J., for supplying all 2,5-dimethyl- and 2-hepta-5-methyl- and part of 2-ethyl-5-methylbenzimidazole; and to Dr. L. D. Abbott, Dept. of Biochemistry, Med. Coll. of Virginia, Richmond, for supplying greater part of 2-ethyl-5-methylbenzimidazole used.

‡ U. S. Public Health Predoctorate Research Fellow supported by Nat. Cancer Inst. Present address: Poultry Science Dept., Ohio State Univ., Columbus.

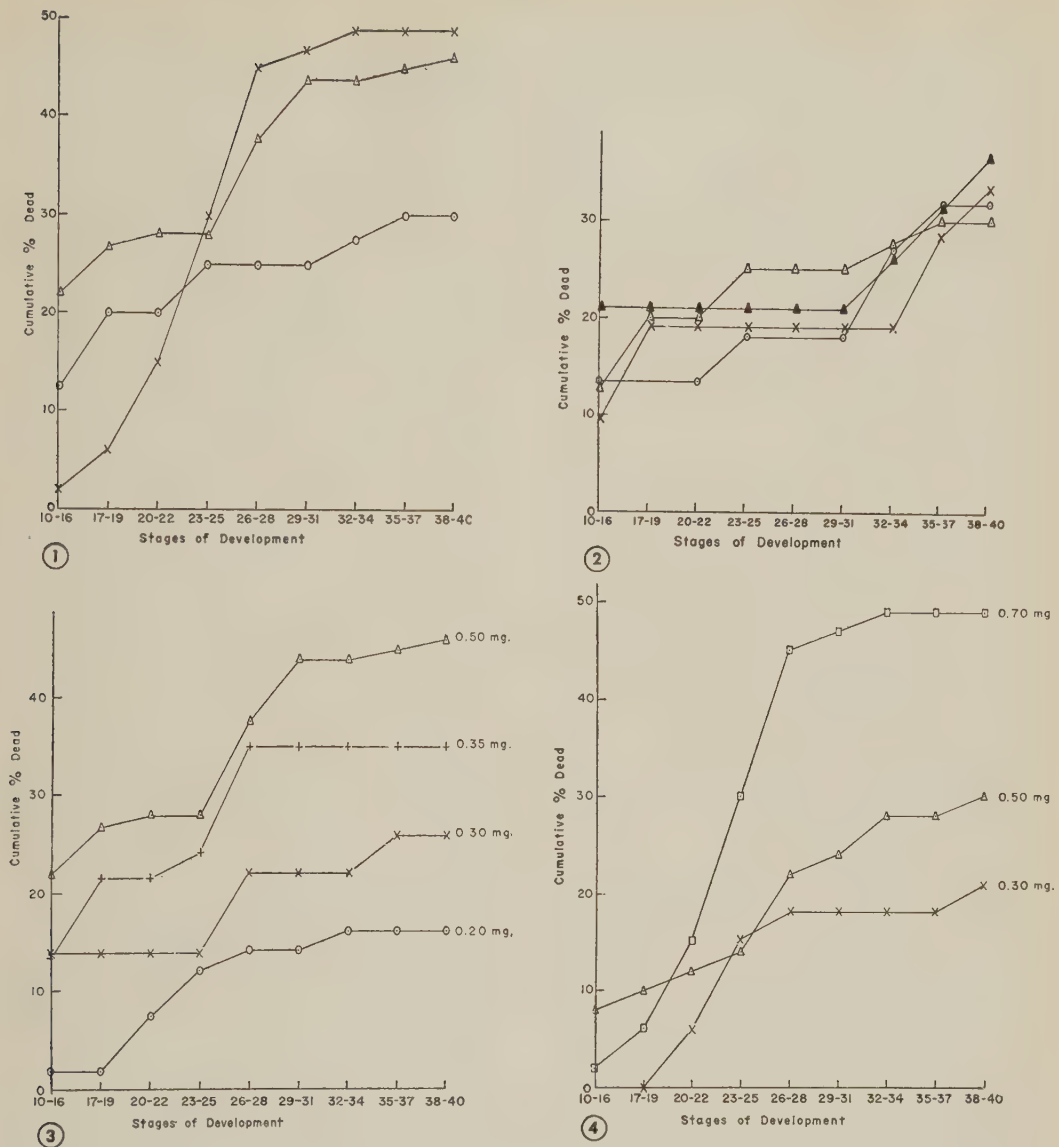


FIG. 1. Inhibition curves of DMB, EMB, and HMB when an approximately equivalent dose was administered to chick embryos prior to incubation. ×——× 3.0 μM HMB (avg of 3 repeats). ▲——▲ 3.1 μM EMB (avg of 5 repeats). ○——○ 3.4 μM DMB (avg of 4 repeats). Ordinate represents stages at which development ceased and abscissa represents cumulative % dead at times indicated. Stages are represented in groups of 3 from stages 17 to 40 (ca. 52-64 hr to 14 days). Stages 10-16 were combined because many embryos ceasing development during this period were too malformed to be adequately staged.

FIG. 2. Inhibition curves of several doses of DMB when administered to chick embryos prior to incubation. ○——○ 0.20 mg; ×——× 0.30 mg; ▲——▲ 0.40 mg; △——△ 0.50 mg. Each curve represents avg of 2-4 repeats. Explanation of graph is given under Fig. 1.

FIG. 3. Inhibition curves of several doses of EMB when administered to chick embryos prior to incubation. Each curve represents avg of 5 repeats. Explanation of graph is given under Fig. 1.

FIG. 4. Inhibition curves of several doses of HMB when administered to chick embryos prior to incubation. Each curve represents avg of 3 repeats. Explanation of graph is given under Fig. 1.

sponding increase in mortality and increase in percentage of embryos ceasing development in the early period. Also, these doses usually did not inhibit development appreciably after stage 28. Dose variation of HMB (Fig. 4) exhibited curve changes both peculiar to itself and similar to EMB. Increase in dose increased mortality and the highest and lowest dose predominately failed to inhibit development after stage 28. However, contrary to EMB, these doses failed also to inhibit development before stage 17. The middle dose inhibited development at almost every period through 40.

B. *Gross malformations.* These inhibitors produced numerous malformations. Table I summarizes malformations according to stage of the embryo at which development ceased. Since most types of malformations were associated with embryos of a limited embryonic age period, it is obvious that the incidence of the different kinds of malformations is reflected in the inhibition curves previously discussed.

Most dead embryos were malformed. However, several dead embryos resulting from highest dose of HMB and lower doses of EMB appeared grossly normal. Those embryos surviving treatments were not affected in any obvious manner. Chicks which hatched in several experiments were kept 2 to 3 weeks and were not different from the controls. Most embryos that ceased development at stages 10-16 and had a heart, were still living at 5-7 days as evidenced by pulsations of the heart. The area vasculosa (A.V.) of the anidian embryo (a well de veloped area vasculosa in the absence of an embryo), vesiculated embryos, and embryos in the form of an unidentified mass of tissue also were generally still living as indicated by redness of blood. Other malformations examined at this period were clearly defined and not degenerated.

Malformations of amnion and chorion were characterized by failure of the amnion to envelop the embryo in part or totally, and in some cases the amnion was completely formed but exceedingly small apparently causing an exaggerated ventral curvature of the embryo. In other cases the embryo was found within the yolk sac possessing no amnion. Many variations of torsion and flexion defects occurred. In some cases the embryo completely turned so that it lay on its right rather than left side or incompletely turned giving it an S-shape. In others, all or part of the embryo failed to turn but continued to lie on its ventral surface, or the head turned downward or upward in relation to yolk, or the embryo was in an E-shape. Flexion disturbances generally involved a failure of posterior trunk to curve ventrally in spite of the fact it lay on its left side. Many embryos killed by HMB exhibited the latter minor malformation. Disproportionate development of trunk and limbs often accompanied lordosis and involved ectro- or micromelia of all limb buds or one or both limb buds on one side, (left wing bud was most often affected), microsomia, or anouria or brachyouria. The "collapsed" optic lobe and eye were characterized by reduction in size of the structures and always occurred on right side and only in embryos of stages 26-31. This may have been a result of morbidity but did not occur in all embryos ceasing development at these stages. Exposed or extruded brain involved complete or partial absence of skull and a mushrooming effect of brain of stage 26-29 embryos. Beak malformations also varied considerably from slight twisting of beak to shortening of lower or an absence of upper beak. Beak malformations resulting from DMB were of the more severe type.

EMB produced the greatest and HMB the least number of different types of malformations (Table I). Malformations found in controls and produced by at least one dose of each inhibitor were: embryo formed as unidentified mass or vesicles (c), heart and trunk with greatly reduced or absent head region (g), affected torsion and flexion (n), microphthalmia (q), and beak malformations (v). Disturbance of torsion and flexion was the only malformation produced by every treatment. It is also evident (Table I) that certain malformations were produced by only DMB and EMB (*e.g.*, k and u) while other malformations were produced by only EMB and HMB (*e.g.*, f, l, and r), and several other

TABLE I. Incidence of Types of Malformations after Injection of Various Doses of DMB, EMB, and HMB into the Albumen Prior to Incubation.

Malformations occurring at different stages	Inhibitors: DMB Dose/egg (mg) .2-.3	EMB .2-.3	EMB .35	EMB .5	HMB .3	HMB .5	HMB .7	Controls
Stages 10-19	% of total malformations							
a. Anidian embryo	3.1	13.3	0	2.7	0	0	0	7.1
b. Small A.V. with embryo	0	6.7	2.7	8.1	0	0	0	0
c. Embryo as vesicles or mass	3.1	0	8.1	24.3	0	7.1	0	7.1
d. Heart—no or reduced head & trunk	0	0	5.4	8.1	0	0	0	0
e. Head—no or reduced heart & trunk	0	0	0	8.1	0	0	0	0
f. Heart + head—no or reduced trunk	0	0	5.4	5.4	0	14.3	0	0
g. Heart + trunk—no or reduced head	28.1	6.7	13.5	0	0	7.1	0	7.1
h. Vesicle in neural canal	0	0	5.4	0	0	0	0	0
Total	34.3	26.7	40.5	56.7		28.5		21.3
Stages 16-31								
i. Edema	0	6.7	2.7	2.7	0	0	0	7.1
j. Amnion-chorion malformation	0	0	2.7	5.4	0	0	0	0
k. Heart malformation	3.1	0	5.4	0	0	0	0	0
l. Visceral arch malformation	0	13.3	0	0	0	7.1	0	0
m. Open post. neural canal	0	0	0	5.4	0	0	0	0
n. Torsion & flexion defect	6.2	6.7	21.6	8.1	57.0	36.0	65.0	14.3
o. Lordosis	0	6.7	2.7	2.7	0	0	0	0
p. Dispropor. dev. of trunk & limbs	0	6.7	5.4	2.7	0	0	0	0
q. Microphthalmia	3.1	0	0	2.7	0	0	10.0	7.1
r. "Collapsed" optic lobe & eye	0	13.3	5.4	2.7	28.0	14.3	25.0	0
s. Other	0	0	0	0	0	0	0	28.6
Total	12.4	53.4	45.9	32.4	85.0	57.4	100	57.1
Stages 28-40								
t. Exposed or extruded brain	15.6	0	5.4	0	0	0	0	7.1
u. " viscera	6.2	13.3	2.7	0	0	0	0	0
v. Beak malformations	28.1	6.7	5.4	10.8	0	14.3	0	14.3
w. Other	3.1	0	0	0	14.0	0	0	0
Total	53.0	20.0	13.5	10.8	14.0	14.3		21.4
No. of fertile eggs	89	37	64	79	33	51	46	286
" " malformations	32	15	37	37	7	14	20	14
% malformations in fertile eggs	36	41	58	47	21	27	43	5

malformations were produced by only EMB (b, d, e, h, j, m, o, and p).

Discussion. This work demonstrates that very small changes in molecular structure of a chemical agent will elicit specific responses from the developing chick embryo, not only quantitatively but also qualitatively different. Mortality was least sensitive of the 3 criteria employed for determining embryonic response. By using mortality, inhibitory effectiveness could be obtained which differentiated between action of DMB and of EMB and HMB, but not between EMB and HMB. However, by determining inhibitory and teratogenic patterns, and in spite of the fact that EMB and HMB had equal lethal action, there was a great difference between inhibition patterns and types of malformations produced by these 2 compounds. Thus, the developing chick embryo responds in a specific manner to equivalent doses of each of the 3 alkyl benzimidazoles. This specificity is contrary to the implication that the action of these benzimidazoles differs only in degree of inhibition in that a characteristic order of inhibition has been found in a variety of biological systems (9,11,12,15,16). However, Tamm(17) found that DMB, EMB, and 2-butyl-5-methylbenzimidazole selectively inhibited to varying degrees, influenza virus multiplication as compared to toxicity to the chorio-allantoic membrane; and Blackwood and Harris(10) found a difference in motility inhibition of chicken sperm by EMB and DMB in relation to their ability to preserve generative powers; thus indicating differences in action other than degree of inhibition. Nevertheless, inhibitory and teratogenic patterns in the embryo indi-

cate that these compounds also have similarities as well as differences in action. One might conclude that these compounds interrupt multiple metabolic processes with some actions common to each compound while others are different. Reversal studies of these and other benzimidazoles indicate that their action(s) is indeed multiple(12,14,18-22).

It seems to be a characteristic of homologous series that members of the series produce distinct yet similar effects. This is seen here as well as in studies of Ambellan(8), who observed the action of mono-, di-, and triphosphorylated nucleosides on amphibian embryos. Other examples of this may be the paradoxical effects of homologues of trimethylalkylamines and several other compounds on neuro-muscular function and other physiological or metabolic processes. These effects of the latter compounds vary according to alkyl substitution; the shorter chains cause only muscle contraction, the longer chains only muscle relaxation, while intermediate chains cause both contraction and relaxation (23).

The numerous types of malformations and variable Vit. B_{12} reversal(12) obtained from the 3 benzimidazoles would seem to support the contentions of multiple action as well as interruption of basic process(es) in metabolism(15). It seems reasonable to assume that these two factors play very important roles in teratogenic action.

Summary. Inhibitory and teratogenic effects of various doses of benzimidazole homologues, 2,5-dimethyl- (DMB), 2-ethyl-5-methyl- (EMB), and 2-hepta-5-methyl- (HMB), were studied in the chick embryo following injection into the albumen prior to incubation. Three criteria of determining embryonic response were employed: lethality, inhibition curves derived from cumulative % embryos which ceased development at progressive stages, and types and incidence of types of malformations. EMB and HMB had about the same degree of lethality and were considerably more lethal than DMB. Inhibition curves from equivalent doses were different for each compound, and dose increase caused a different pattern of change in inhibition curves; thus, the embryo responds specifically to equivalent doses of each compound. Certain types of malformations resulted from all inhibitors, others resulted from only DMB and EMB, others from EMB and HMB, and still others from only EMB. The incidence of each different type of malformation was reflected in inhibition curves since most types of malformations were associated with embryos of a limited embryonic age period. Although there were differences in response to the compounds, there were also similarities.

1. Landauer, W., *J. Cell. Comp. Physiol.,* 1954, v43, Suppl. 1, 261.

2. Ancel, P., *J. Embryol. Exp. Morph.,* 1955, v3, 335.

3. Liedke, K., Engleman, M., Graff, S., *J. Exp. Zool.,* 1954, v127, 201.

4. ———, *ibid.,* 1957, v136, 117.

5. ———, *J. Emb. Exp. Morph.,* 1957, v5, 368.

6. Bieber, S., *J. Cell. Comp. Physiol.,* 1954, v44, 11.

7. Waddington, C. H., Feldman, M., Perry, M. M., *Exp. Cell. Res.,* 1955, Suppl. 3, 366.

8. Ambellan, E., *J. Embryol. Exp. Morph.,* 1958, v6, 86.

9. Hendlin, D., Soars, M. H., *J. Bact.,* 1951, v62, 633.

10. Blackwood, U. B., Harris, G. C., Jr., Proc. Soc. Exp. Biol. and Med., 1960, v103, 60.

11. Tamm, I., Folkers, K., Shunk, C. H., Heyl, D., Horsfall, F. L., *J. Exp. Med.,* 1953, v98, 245.

12. Blackwood, U. B., Shorb, M. S., Proc. Soc. Exp. Biol. and Med., 1958, v99, 232.

13. Hamburger, V., Hamilton, H. L., *J. Morph.,* 1951, v88, 49.

14. Blackwood, U. B., Doctorate Thesis, Univ. of Maryland, 1958.

15. Abbott, L. D., Dodson, M. J., Auvil, D. K., *Fed. Proc.,* 1956, v15, 674.

16. Billett, F., *Experimentia,* 1958, v16, 99.

17. Tamm, I., *J. Bact.,* 1956, v72, 42.

18. Nakamura, M., Proc. Soc. Exp. Biol. and Med., 1957, v95, 524.

19. Funk, H. B., Nathan, H. A., *ibid.,* 1958, v99, 394.

20. Woolley, D. W., *J. Biol. Chem.,* 1944, v152, 225.

21. Clayton, C. C., Proc. Soc. Exp. Biol. and Med., 1958, v97, 510.

22. Brown, E. G., *Biochem. J.,* 1958, v70, 313.

23. Ariens, E. J., Simonis, A. M., DeGroot, W. M., *Arch. Inter. Pharm. Therap.,* 1955, v100, 298.

Received March 11, 1960. P.S.E.B.M., 1960, v104.

Role of Histamine in Endotoxin Shock.* (25843)

LERNER B. HINSHAW,† JAMES A. VICK,‡ CURTIS H. CARLSON AND YOU-LING FAN
Depts. of Physiology, Pharmacology and Physiological Chemistry, University of Minnesota, Minneapolis

Our recent work has been concerned with hemodynamic changes in animals following lethal injections of endotoxin(1-6). Similarity between vascular actions of histamine and endotoxin has been noted(4-7), suggesting that hemodynamic changes in endotoxin shock may be brought about by release of histamine. Our aim in this study was (a) to estimate changes in whole blood and plasma histamine following lethal injections of endotoxin, and (b) to determine if changes in vascular reactivity to histamine may result following endotoxin administration. Our findings suggest a prominent role of histamine in shock produced by endotoxin.

Methods. Histamine estimations were made with chemical procedure utilizing paper chromatography. Blood samples from catheterized femoral vessels of dogs, anesthetized with sodium pentobarbital (30 mg/kg), were collected directly into 4 volumes of acetone. Plasma samples were obtained from chilled centrifuged blood containing 1.7% potassium oxalate(8), plasma then added to acetone as above. Samples were refrigerated 12 hours, filtered until clear and evaporated to dryness, by warming and later by lyophilization. Dried residue was extracted with water and evaporated to standard volume < 1 ml. Equal aliquots of final solutions were placed on Whatman #1 paper and run for 10-16 hours in a solvent of n-butanol:acetic acid: water (4:1:5), using descending paper chromatography. Pauly reagent, followed by sodium carbonate overspray was used for color development(9). R_f's ranged between 0.10-0.14, and recoveries were 90-95% complete. Other procedures(10,11) were used for comparison with present technic and results were in close agreement. Changes in histamine concentration were estimated by comparisons of area X density of spots to pre-endotoxin values. Absolute concentrations of blood histamine were also estimated by comparisons to known standard concentrations, and all values before and after endotoxin ranged between 0.03 and 2.20 μg/ml. *E. coli* endotoxin (Difco), 2 mg/kg, was administered intravenously to anesthetized adult dogs. In second series, hindlimbs of dogs were perfused with heparinized blood continuously obtained from adult anesthetized dogs, as previously described(12). Intra-arterial injections of histamine (1 μg) were made in the isolated limb before and after intravenous injection of *E. coli* endotoxin (2 mg/kg, Difco) into the intact dog. Limbs were suspended on strain gauge weighing device and perfused at highest flow compatible with the isogravimetric state, flow remaining constant until termination of experiments.

Results. In Fig. 1 mean changes in plasma and whole blood histamine are shown following lethal injections of endotoxin. Within 2 minutes after injection there is a marked fall in whole blood histamine and a rise in plasma histamine. Plasma histamine remains

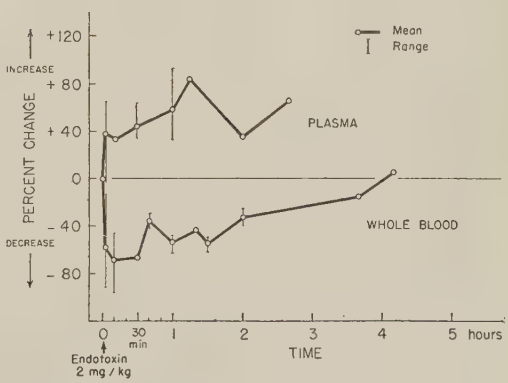


FIG. 1. % changes in histamine in 12 dogs following intrav. inj. of *E. coli* endotoxin (2 mg/kg). (6 dogs, plasma histamine, upper curve; 6 dogs, whole blood histamine, lower curve.) Mean values shown from zero time to termination of experiment.

* Research supported by grants from Minn. Heart Assn. and USPHS.

† Research perfcrmed during Lederle Medical Faculty Award, 1959-1962.

‡ Research done during appointment as NIH Fellow.

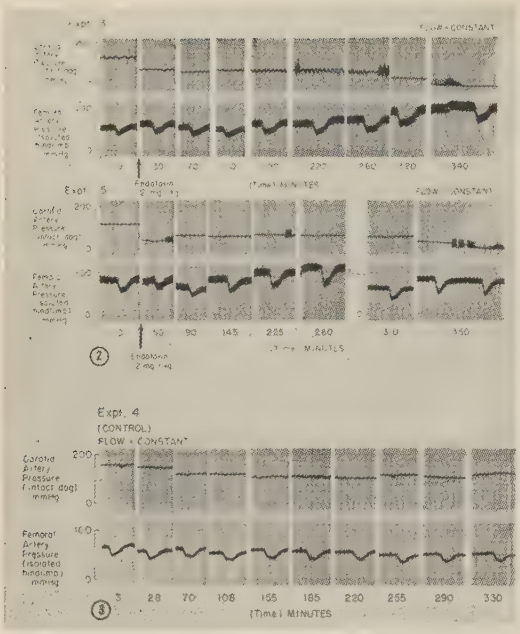


FIG. 2. Poly-viso recorded data from 2 separate whole dog-limb perfusion experiments (total 6 experiments). Responses of limb artery pressure to inj. of histamine are shown on lower panel of each section. Experiments terminated at death of animals.

FIG. 3. Control whole dog-limb perfusion experiment, as in Fig. 2, except that endotoxin is not administered.

increased during post-endotoxin period while whole blood histamine gradually returns toward pre-endotoxin level.

Recorded data from 2 separate limb perfusions are shown in Fig. 2. Vascular responses to injected histamine become progressively greater during post-endotoxin period, as contrasted with separate control experiment (Fig. 3) in which endotoxin is not administered and where no significant changes in histamine response are observed. In final 2 experiments (Fig. 4), comparisons are made of response to histamine when endotoxin and epinephrine (Suprarenin) are administered separately (flow constant). The rise in limb vascular resistance following injection of endotoxin (also seen in Fig. 2) is reproduced by constant infusion of epinephrine, 1-2 μg/min. A major difference exists between the 2 experiments, in that decrease in resistance to histamine becomes progressively greater after endotoxin, but less during infusion of epinephrine.

Discussion. Although there are limitations to the chemical procedure for histamine analysis(8,13,14), errors in the method do not invalidate measurements of *changes* in histamine(8) under special consideration in the present study. Changes in histamine concentration following lethal injections of endotoxin may be explained on the basis of release of histamine from the bound form in whole blood to the circulating form in plasma (15,16), or in addition, from histamine possibly released from tissue such as muscle(14). Our findings are consistent with results obtained from experiments concerned with anaphylaxis(16) and endotoxin(14), and further substantiate that the canine response to endotoxin may be anaphylactoid in type(17).

Progressive rise in limb vascular resistance after endotoxin may have occurred in response to the dual effect of increase in plasma histamine and increased responsiveness of certain vascular beds to circulating histamine. These additive effects could also explain the reported decreases in vascular resistance after endotoxin in various vascular beds(5,6). The implications of these findings support a hypothesis recently proposed by Schayer(18) regarding probable role of histamine in shock.

Summary. Intravenous injection of lethal doses of endotoxin in anesthetized dogs results in rapid decrease in total blood hista-

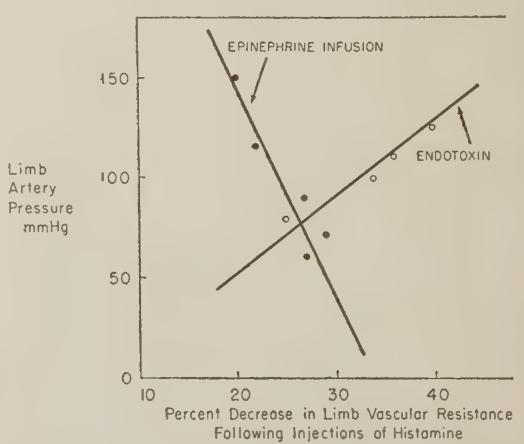


FIG. 4. Whole dog-limb perfusion experiments. Responses to histamine, as in Fig. 2, 3, following inj. of endotoxin and during infusion of epinephrine (2 separate experiments). Progressive rise in vascular resistance occurred in both instances. Decrease in resistance to histamine becomes progressively greater after endotoxin, but less during infusion of epinephrine, as vascular resistances are increasing.

mine, a concomitant rise in plasma histamine, and increased peripheral vascular responsiveness to histamine. These findings support the view that histamine performs a crucial role in progressive development of hypotension following administration of endotoxin.

Appreciation is expressed to Lorentz E. Wittmers for technical assistance.

1. MacLean, L. D., Weil, M. H., *Circ. Res.,* 1956, v4, 546.

2. Weil, M. H., MacLean, L. D., Visscher, M. B., Spink, W. W., *J. Clin. Invest.,* 1956, v35, 1191.

3. Hinshaw, L. B., Gilbert, R. P., Kuida, H., Visscher, M. B., *Fed. Proc.,* 1958, v17, 71.

4. Kuida, H., Hinshaw, L. B., Gilbert, R. P., Visscher, M. B., *Am. J. Physiol.,* 1958, v192, 335.

5. Hinshaw, L. B., Gilbert, R. P., Kuida, H., Visscher, M. B., *ibid.,* 1958, v195, 631.

6. Hinshaw, L. B., Bradley, G. B., Carlson, C. H., *ibid.,* 1959, v196, 1127.

7. Gilbert, R. P., Hinshaw, L. B., Kuida, H., Visscher, M. B., *ibid.,* 1958, v194, 165.

8. Lowry, O. H., Graham, H. T., Harris, F. B., Priebat, M. K., Marks, A. R., Bregman, R. U., *J. Pharm. Exp. Therap.,* 1954, v112, 116.

9. Ames, B. N., Mitchell, H. K., *J. Am. Chem. Soc.,* 1952, v74, 252.

10. Barsoum, G. S., Gaddum, J. H., *J. Physiol.,* 1935, v85, 1.

11. Code, C. F., *ibid.,* 1937, v89, 257.

12. Hinshaw, L. B., Gilbert, R. P., Kuida, H., Visscher, M. B., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 684.

13. Code, C. F., *Physiol. Revs.,* 1952, v32, 47.

14. Kun, E., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 197.

15. CIBA Foundation Symposium on Histamine, Little, Brown & Co., Boston, 1956, 36.

16. Waalkes, T. P., Weissbach, H., Bozicevich, J., Udenfriend, S., *J. Clin. Invest.,* 1957, v36, 1115.

17. Weil, M. H., Spink, W. W., *J. Lab. & Clin. Med.,* 1957, v50, 501.

18. Schayer, R. W., *Science,* 1960, v31, 226.

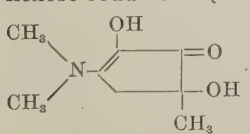
Received March 25, 1960. P.S.E.B.M., 1960, v104.

Circling Syndrome Produced in Mice by Dimethylaminohexose Reductone. (25844)

WINDSOR CUTTING, ARTHUR FURST, DOROTHY READ, GEORGE READ AND HENRIETTE PARKMAN

Dept. of Medical Microbiology, Stanford University, Calif.

That certain amino-sugar reductones produce circling phenomena in rats, the movements suggesting those seen in waltzing mice, will shortly be reported by Ambrose, Robbins and DeEds of the U. S. Dept. of Agriculture who made the initial observations. After continued feeding the rats circled rapidly lasting many minutes, often set off by such stimuli as a bright light. Pulmonary hemorrhages and thyroid hyperplasia were observed but no primary central nervous or labyrinthine lesion was found. Lack of body fat, presumably from excessive exercise, was characteristic. In this paper reactions produced in mice by one of these reductones, dimethylaminohexose reductone (hereafter called reductone)



are reported.

Results. 1. *Induction of circling.* The circling syndrome was easily produced in white Webster mice by reductone. Table I shows effect of route and dosage on circling. Administration in drinking water or by injection (subcutaneous or intraperitoneal in distilled water or saline) was effective. Maximal effect was obtained after a single injection of 5 mg/20 g mouse, or 4 daily injections of 2.5 mg. At higher dosages early deaths occurred in some animals, and at lower dosages the syndrome was absent or incompletely developed.

2. *Circling syndrome.* Reductone symptoms were noticeable 3 to 4 hours after administration. The first, and most characteristic sign was head tossing, an upward jerk or shake repeated rapidly, accompanied by obvious difficulty in coordination and balance.

TABLE I. Effect of Dimethylaminohexose Reductone Administration to Mice.

No. of mice	Daily dose	No. days admin.	Route	Results
10	.1%	60	In drinking water	Whirling in 24 hr; 4 deaths in 2 mo
6	.6 mg	1	Intraper.	No effect
6	1.25	1	"	*Idem*
10	2.5	1	"	Some head tossing; poor circling
10	5.0	1	"	Rapid head tossing; maximal circling
100	5.0	1	Subcut.	*Idem*
10	7.5	1	Intraper.	"
10	10.0	1	"	" , but 2 deaths in 2 days
6	.6	4	"	Slight head tossing if mouse excited
6	1.25	4	"	Moderate head tossing
6	2.5	4	"	Rapid head tossing; good to maximal circling
6	5.0	4	"	Rapid head tossing; maximal circling

At almost the same time periodic circling began. This consisted of running in tight circles, in either direction, for periods of seconds to several minutes. Periodic maximal episodes of circling occurred within a few hours and continued, though with a gradual lessening of accompanying incoordination, for the lifetime of the mouse, even after a single injection. Head tossing and circling periods could often be induced by noise, handling, bright light, or new surroundings. When not so stimulated the mice tended to be rather quiet, huddling in a group. Several less striking characteristics were also noted. The mice seemed generally more active than controls. They did not build hills or nests in the sawdust on floor of cage but kept it flat, apparently from circling and running. A pregnant reductone mouse, however, if put alone, usually built a good nest. The backs of male animals became scarred, and in places hairless, from backbiting. When tested for swimming ability, these mice still circled, some swimming in circles on the surface, while others consistently dived under the water to circle on a vertical plane. These latter would drown if allowed to do so.

3. *Weight changes.* During first 5 days after administration of reductone, the mice lost as much as 25% of body weight. Although they were seen to eat, their head jerking appeared to interfere when they raised their heads to drink from overhead tubes and water consumption was greatly reduced. By 7 days they seemingly learned to compensate for the head bobbing and had begun to regain weight, and by 2 weeks had reached their original weight. Striking thinness, as described for rats was not characteristic, and an occasional mouse became somewhat obese. A group of 10 mice, 6 months after induction of reductone syndrome, weighed between 26 and 34 g, a range similar to that for normals of same age. Although food consumption was not measured, the impression was that they ate more food than normal mice.

4. *Effect on body temperature.* Rectal temperatures of 26 mice were taken 6 months after administration of reductone, using thermocouple previously described(1). Average temperature, 40.5°C, did not vary significantly from that of similar number of normal controls, 41.0°C. The periodic increased activity did not produce sustained increase in temperature.

5. *Oxygen consumption.* Oxygen consumption was measured by a simple method(1) in mice 6 months after reductone administration. Measurements were made during quiet periods when mice were not circling or actively tossing their heads. Average time for consumption of 4 cc of oxygen was 3.6 ($\pm$ 0.75 SD) and 3.5 ($\pm$ 0.76) seconds/g body weight respectively for 12 reductone mice and 11 control mice of similar weight. It is apparent that resting reductone mice do not have a higher oxygen consumption than normals.

6. *Quantification of activity.* The general activity of reductone mice was compared to that of normals on activity table previously described(1). Ten normal mice had average activity table count of 678 recorded move-

TABLE II. Effect of Stress on Normal and Reductone Mice, the Latter Showing Slight Impairment on Turntable and Marked Impairment on Rotating Rod.

Wk since reductone (5 mg/mouse subcut.)	No. of mice	% success in 15 trials	
		Turntable	Rotating rod
Controls	80	96 ± 3*	92 ± 7*
4	10	84 ± 5	13 ±12
12	"	93 ± 5	43 ± 5
16	"	100 ± 0	50 ± 7

* ± S.D.

ments in the standard 4 minute test. Each was then given reductone, 5 mg subcutaneously, and tested 7 and 8 days later, when there was no sustained circling. Average activity counts were 736 and 674, indicating a lack of change in activity (when there was no circling). Minor head tossing was not of magnitude great enough to activate the activity table counter. When animals were tested during active circling, counts rose to high levels. Thus 2 mice that circled almost constantly during the test, produced counts of 3398 and 2879, one that spun intermittently had a count of 1137, but one that spun only once or twice gave a count of 722, *i.e.*, in the average range. Thus the basic (non-circling) activity registered on this apparatus was not changed by reductone administration. Periods of increased ordinary movement were obviously balanced by periods of subnormal activity in a 4 minute period. All-out spinning produced a high activity count during circling.

7. *Response of reductone mice to stress* was studied on the turntable and 3 cm rotating rod(1). Each mouse was tested 15 times. Data are recorded as percent of successful tries ± SD. Results are shown in Table II. Reductone does not interfere greatly with successful riding of the turntable, but interferes seriously, though decreasingly as weeks pass, with performance on the rotating rod.

8. *Gross pathological findings.* Mice were sacrificed daily for 12 days after administration of reductone. Diminution in body fat was noticeable on fourth day, but largely rectified by twelfth day. Lung hemorrhages, prominent in rats, were minor and occurred similarly in controls and reductone mice.

Discussion. The effectiveness of dimethylaminohexose reductone in producing a characteristic activity in mice prompts a comparison with other types of similar activity. The type most studied is undoubtedly that associated with genetic abnormalities and often called "waltzing"(2,3).

The "Japanese waltzer" with a *V waltzing* gene has a characteristic syndrome which consists of head shaking, running in circles, deafness and inability to swim. The pathology underlying this behavior is incompletely understood, but degeneration of inner ear structures within the first few weeks of life has been reported and degeneration of all acoustic tracts in the brain has been noted. Waltzing behavior characterized by choreic head movements, circling and inability to swim in varying degree, has also been found in other genetically independent strains, as follows: a. *Sh-1 shaker-1*—after 2-4 weeks degenerative changes of inner ear and possible atrophy of corpus callosum, deafness, nervous rapid up and down movements of the head; b. *Sh-2 shaker-2*—pathology not studied, deafness and movements indistinguishable from type above; c. *St shaker short*—embryological failure of development of semicircular canals and endolymphatic appendages; cochlea and cortical organs abnormal; deafness, disturbances of equilibrium as in shaker 1; d. *Kreisler*—resembles shaker-short mice; e. *Jerker and pirouette*—pathology not studied, deafness; f. *Fidget*—pathology not known, similar in behavior but do not become deaf. Some of these names may be duplicates as the genetic characteristics are not known in all cases. Some mice not ordinarily considered waltzers show abnormal excitability in youth. This is particularly true of the C-57 Black line; males of the NB strain are aggressive and show characteristic tail rattling. Also somewhat similar are seizures which can be produced by audiogenic stimuli, particularly in the DBA strain.

The effects of drugs on genetic waltzing were studied by Rothlin and Cerletti(4) who reported that lysergic acid, mono- or di-ethyl amide decreased waltzing in congenitally waltzing mice while at the same time increas-

ing general activity. A preparation of dihydroergot alkaloids (Hydergin) decreased circling and showed a generally sedative effect, while ergotamine and dihydroergotamine did not influence activity.

Temporary or permanent waltzing behavior in mice similar to that of genetic waltzers has been produced by a number of drugs. According to Goldin(5), Ehrlich reported that arsacetin was active in this respect. Goldin also reported waltzing from dialkyl and heterocyclic derivatives of β-chloroethylamine. Pathological studies of the mice revealed destruction of cells of the granular layer of the lingula of the cerebellum within 2 to 5 days after injection and also small areas of gliosis in brain stem, medulla and upper cord. Inner ear and auditory nerve appeared undamaged.

Athough β-aminoproprionitrile, the poison found in sweet peas which causes lathyrism, did not cause waltzing, the bis form has been reported to produce the waltzing syndrome. Thus Hartman and Stitch(6) showed that after doses of 1.5 to 2.0 g/kg of bis aminoproprionitrile (bis-β-cyanoethylamine; β β′-iminodiproprionitrile) abnormal movements began in about 3 days and lasted many days with characteristic running in circles, moving backwards, twitching and a general resemblance to waltzing mice. Rudberg(7), using somewhat smaller doses, 0.75 to 1.5 g/kg, observed a maximal amount of waltzing in 8 to 15 days which continued for more than 3 months. The pathological changes were described by Bachuber(8) as destruction of Purkinje cells in the cerebellum, and of anterior horn ganglion cells in the spinal cord. The hyperactivity caused by bis-β-aminoproprionitrile was reported by Azima and Grad (9) to be temporarily controllable with reserpine, by Widlocher *et al.*(10) with lysergic acid diethylamide and by Selye(11) to be prevented by thyroxin. (We have not been able to show a similar thyroxin effect on mice given reductone.) Rudberg(7) reported similar effects from chlorpromazine, but noted that hyperactivity was not affected by Hydergin or ergotamine tartrate.

Scholler(12) reported that methylphenyltriazine in doses of 0.5 g/kg intraperitoneally or 1 g/kg orally in mice gave impairment of righting reflexes within 2 hours, followed by depression and death. Half these doses gave head bobbing and inability to swim well, such as characterize waltzing mice.

Another type of abnormal movement, somewhat different from waltzing, was reported by Siegmund, Cadmus and Lu(13), as a writhing syndrome, which followed administration of 2-phenyl-1, 4-benzoquinone in dose of 1 mg/kg intraperitoneally. Various analgesics exerted a suppressive effect. In our laboratory these writhing symptoms appear to be at least in part the result of peritoneal irritation, since vehicles alone such as 0.3% carboxymethylcellulose give similar effects.

The effects produced by reductone resemble those seen in genetically waltzing mice, and those described by the substituted β-chloroethylamines and bis-β-amino-proprionitrile, and to some extent those produced by methyl-phenyltriazine. However, pathological lesions in the central nervous system or in the labyrinthine or auditory portions of the ear have not been observed. The characteristic syndrome after reductone shows all signs associated with waltzing except deafness, although impairment in swimming may be less severe. Although reductone gave similar effects in rats and mice, limited trials in goldfish produced only questionable abnormalities.

Conclusions. 1. A permanent circling syndrome can be produced in mice by a single injection of dimethylaminohexose reductone. 2. The syndrome is characterized by frequent head tossing and running in circles. Body temperature, oxygen consumption and basic activity are not altered but ability to resist stress by staying on a rotating rod is decreased.

1. Cutting, W., Parkman, H., Read, G., Read, D., Cutting, J., Furst, A., *Arch. int. Pharmacodyn.*, 1959, v121, 14.

2. Gruneberg, H., *Bibliographica Genetica,* 1952, Martinees Niijhof, The Hague, p178.

3. *Biology of the Laboratory Mouse,* 1956, G. D. Snell, Ed., Dover, N. Y., p283.

4. Rothlin, E., Cerletti, A., *Helv. Physiol. et Pharmacol. Acta,* 1952, v10, 319.

5. Goldin, A., Noe, H. A., Landing, B. H., Shapiro,

D. M., Goldberg, B., *J. Pharm. Exp. Therap.*, 1948, v94, 249.

6. Hartman, H. A., Stitch, H. F., *Science*, 1957, v125, 445.

7. Rudberg, T., *Acta Pharmacol. Toxicol.*, 1957, v13, 233.

8. Bachuber, T. E., Lalich, J. J., Angevine, D. M., Schilling, E. D., Strong, F. M., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 294.

9. Azima, H., Grad, B., *Fed. Proc.*, 1956, v15, 17.

10. Widlocher, D., Nakajima, H., Thullier, J., *C. R. Soc. Biol., Paris*, 1957, v151, 668.

11. Selye, H., *J. Clin. and Exp. Psychopath.*, 1958, v19, 97.

12. Scholler, J., Philips, F. S., Sternberg, S. S., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 491.

13. Siegmund, E., Cadmus, R., Lu, B., *ibid.*, 1957, v95, 729.

Received March 28, 1960. P.S.E.B.M., 1960, v104.

Urinary Sulfate Ion as a Naturally Occurring Inhibitor of DNase II Activity.* (25845)

ELIZABETH C. RAUENBUSCH AND KURT I. ALTMAN (Introduced by H. A. Blair)
Division of Experimental Radiology, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

It was reported(1) that human urine inhibits its enzymatic activity of desoxyribonuclease II (DNase II) and activates DNase I slightly. The inhibitory material was dialyzable, thermostable and was not retained by anion and cation exchange resins. In the experiments to be described, an attempt was made to establish the chemical identity of the urinary inhibitor of DNase II.

Methods. 1. *Materials.* a. *Desoxyribonuclease II.* DNase II was prepared from calf spleen using the first 3 steps of the preparative procedure of Koszalka *et al.*(2). The sediment obtained in Step 3 was dissolved in a small volume of water and dialyzed overnight against running tap water at room temperature. In those experiments concerned with effect of inorganic ions on enzymatic activity, the preparation was dialyzed against 0.0001% aqueous sodium versenate. b. *Desoxyribonucleic acid.* DNA of salmon sperm was used as purchased from Calif. Fn. for Biochemical Research. c. *Urinary inhibitory material.* 890 ml of freshly voided human urine was dialyzed overnight against 1,080 ml of distilled water. The resulting 960 ml of dialysate was concentrated to 265 ml by lyophilization. In some experiments, the concentrated dialysate was passed subsequently through a column of activated charcoal (80 mesh). d. *Ashing of inhibitory material.* To determine whether the inhibitor removed by the ion-exchange resins was organic or inorganic in nature, the solid residue remaining after evaporation of solvent from the charcoal-treated urinary dialysate was ashed by heating to redness in a porcelain crucible over a Bunsen burner. The white ash so formed was dissolved in 1 N HCl and the resulting solution evaporated to dryness repeatedly, adding water after each evaporation. The residue was then dissolved in a volume of water equal to that of initial concentrated urinary dialysate. 2. *Analytical procedures.* a. *Assay of DNase II activity.* Enzymatic activity was determined by an adaptation of the procedure for DNA determination described by Schneider and Hogeboom(3). Unless stated otherwise, the following test system was employed:

Acetate buffer, 0.2 N, pH 5.2	2.0 ml
DNA, 0.4% solution	.5 "
Distilled water	1.0 "
DNase II	.2 "

This mixture was incubated for one hour at 37°C and then deproteinized with 1 ml of 2.88 M trichloroacetic acid (TCA). Simultaneously, a similar mixture, lacking DNA, was incubated and then deproteinized with TCA. To the latter deproteinized reaction mixture, 0.05 ml of a 0.4% solution of DNA

* This work was supported, in part, by a grant from the U. S. Atomic Energy Commission.

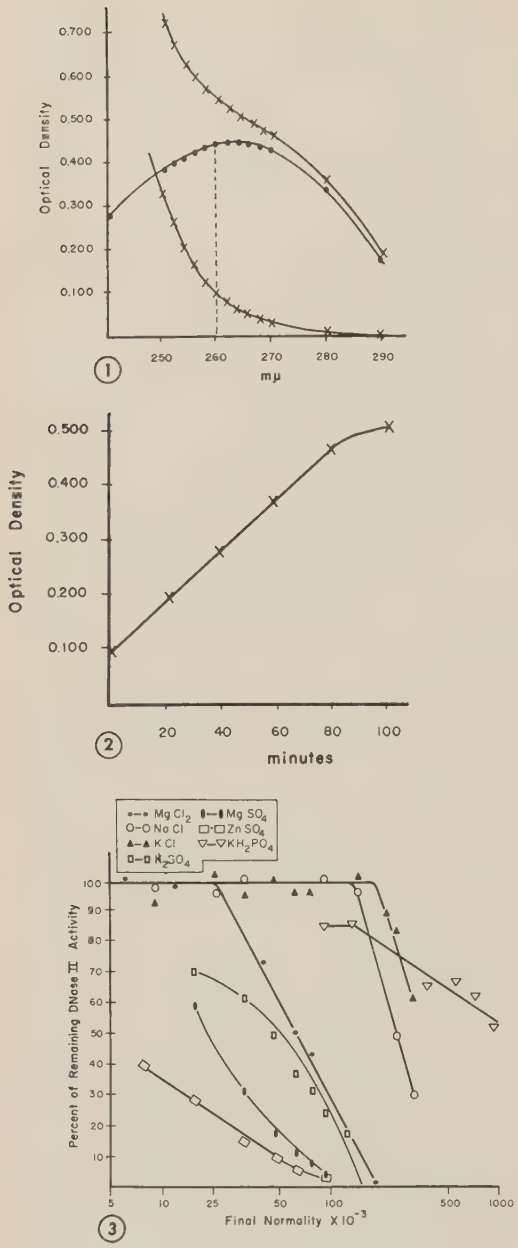


FIG. 1. Optical densities of incubation mixture as a function of wave length. Top curve represents incubation mixtures incubated with DNA. Bottom curve shows a similar plot for an incubation mixture devoid of DNA. Intermediary curve is a representation of the difference between top and bottom curves. Point of maximal difference is designated by broken guide line.

FIG. 2. Change in optical density as a function of time of incubation.

FIG. 3. Inhibitory effect of several cations and anions on activity of DNase II as a function of concentration of the inorganic ions. Concentration (normality) is plotted on logarithmic scale.

was added. The TCA-insoluble material present in both mixtures was removed by centrifugation and the supernatant solutions were diluted 20-fold with water. Subsequently, optical densities of the supernatant solutions were determined at 260 μ using a Beckman Spectrophotometer, Model DU. The enzymatic activity is expressed in terms of the difference between optical density of the complete system and that of the system devoid of DNA. The wave length used to measure changes in DNA as a result of enzymatic action was chosen on the basis of data shown in Fig. 1. In this figure, optical densities of supernatant solutions obtained after deproteinizing the reaction mixtures incubated either with or without DNA are plotted as a function of wave length for the region from 240 to 290 mμ and are represented by the top and bottom curves, respectively. The difference between these 2 optical density values for each wave length studied is shown as in the central curve. The point of maximal difference located at 260 mμ was used in DNase assays. Time of incubation was always one hour. Fig. 2 shows the relationship between change in optical density and time of incubation of the test systems. b. *Evaluation of inhibition of DNase II.* Degree of inhibition of DNase II is expressed as percent of remaining DNase II activity. The reaction mixture was incubated for one hour at 37°C. It had a composition similar to that of the complete test system for DNase II activity, but contained 1.0 ml of the urinary dialysate or of other solutions to be tested for inhibitory activity, instead of the distilled water added in the usual DNase II assay. The difference between enzyme activities in presence and absence of inhibitory material could then be evaluated in terms of percent of remaining DNase II activity. When the effect of the ionic milieu on DNase II activity was investigated, the composition of the system employed to test for inhibitory activity was modified by a) replacing urinary dialysate with 1 ml of a solution containing varying concentrations of solute, and b) by using 1.5 ml of acetate buffer instead of 2.0 ml.

Results. The effects on DNase activity of

TABLE I. Degree of Inhibition of DNase II Activity by Dialysates of Urine.

Materials tested	% of remaining enzymatic activity
Complete test system (without urinary dialysate)	100
Freshly voided urine	83
Concentrated urinary dialysate	67
Idem after treatment with charcoal	78*
Concentrated urinary dialysate†	(a) 84 (b) 76
Ashed dialysate†	(a) 79 (b) 81

In all assays 1.0 ml of solution containing inhibitory activity was used.

* This value is an avg of 3 different preparations.

† (a) and (b) represent determinations on 2 different experiments in which the dialysates are compared before and after ashing.

freshly voided urine and of concentrated urinary dialysate with or without subsequent charcoal treatment are shown in Table I; for comparison, DNase II activity without addition of inhibitory material is also given. Charcoal treatment of the concentrated urinary dialysate had no effect on inhibitor activity. Although it would appear that a considerable fraction of the inhibitory activity was lost on dialysis, this aspect remains uncertain since completeness of dialysis as well as possible interference with the inhibitor assay of other substances in crude urine could not be ascertained satisfactorily.

After charcoal treatment the inhibitory material in urinary dialysate had an affinity for certain ion exchange resins and in this respect appears to differ from dialysates not treated with charcoal since it had been observed earlier(1) that inhibitory material present in untreated dialysate passed through various ion exchange columns. The effect of charcoal treatment is probably due to removal by charcoal of substances which are exchanged more readily by the resin than is the inhibitory material. When charcoal-treated dialysate was passed in succession through 2 ion exchange columns, *i.e.*, Dowex 50 (H+) and Dowex 1 (OH–), it was observed that the effluent solution no longer inhibited DNase II.

Ashing had no detectable effect on inhibitory activity of the serum sample under the conditions of study (Table I). Ability of the inhibitory material to withstand high temperature suggests that the activity is due to inorganic ions normally present in urine. To obtain further evidence for this interpretation, inhibitory effect of various cations and anions on DNase II activity was studied. Confirming observations of Sinsheimer and Koerner (4) and of Shack(5) divalent ions had a stronger inhibitory effect than monovalent ions (Fig. 3). To obtain further quantitative information concerning concentration of ions most likely to cause inhibition in human urine, aliquots of charcoal-treated dialysates taken before and after ashing were analyzed for Ca^{++}, Mg^{++}, phosphate and sulfate.† Only sulfate ions were present in significant amounts. Although most of the sulfate ions were present in the dialysate before ashing, a small, additional amount must have been formed as a result of ashing of organically-bound sulfates or of organic sulfur compounds present in urine.

Assuming that sulfate ions are largely responsible for the inhibitory activity in urinary dialysates, one may calculate the expected degree of DNase inhibition from concentration of sulfate ions known to be present. By applying the inhibition curve obtained for K_2SO_4 (Fig. 3), the expected inhibitory activity corresponding to concentrations of sulfate found before and after ashing was calculated with the following results (average from 2 experiments):

	% of remaining enzyme activity	
	Calculated	Observed
Concentrated urinary dialysate after charcoal treatment	73	78
Ashed dialysate	86	81

The data presented above demonstrate that the order of magnitude of predicted degree of inhibition is in reasonably good agreement with average inhibition found experimentally. The experimentally obtained value of inhibition may be only an approximate value because, in the range of inhibition determined, sensitivity of the test system may be insufficient for detection of small variations in

† Analytical determinations were carried out by Schwarzkopf Microanalytical Lab., Woodside, N. Y.

inhibitor activity. Thus, inhibition of 15%-20% involves uncertainty factors of an order which would make it difficult to compare accurately the degree of inhibition obtained.

Excretion of total sulfur and phosphate (as P) in "normal" human urine is reported as approximately 1.0 g and 1.1 g, respectively, per day(6). Inasmuch as sulfate ions constitute 92% of total sulfur excreted daily(6), it is conceivable that the number of sulfate ions present in the urinary dialysate are sufficient to produce the described inhibitory effect. Quantity of sulfate ions in concentrated urinary dialysates is approximately 8.0 mg/ml or 8×10^{-2} M. This concentration of sulfate was found to inhibit DNase II effectively (Fig. 3).

The data (Fig. 3) suggest that in charcoal-treated urinary concentrates sulfate ions might be largely responsible for inhibition of DNase II. Phosphate ions probably contribute little to inhibition of DNase II in the case of the preparations used since removal of phosphate by charcoal did not affect degree of inhibition. Thus, although phosphate ions may have an inhibitory effect, their contribution to inhibition under our experimental conditions (Table I) appears to be small.

Summary. 1. Urinary dialysates were found to retain inhibitory activity toward DNase II after ashing. 2. Chemical analysis of the charcoal-treated dialysate before and after ashing indicated presence of detectable quantities of sulfate ions but not of Mg^{++} and Cu^{++}. 3. Various divalent ions, in contrast to monovalent ions, had a marked inhibitory effect on DNase II. 4. The data suggest that a major portion of urinary inhibitory activity with respect to DNase is due to presence of sulfate ions in the concentrated urinary dialysate.

1. Kowlessar, O. D., Okada, S., Potter, J. L., Altman, K. I., *Arch. Biochem. Biophys.,* 1957, v68, 231; Koszalka, T. R., Schreier, K., Altman, K. I., *Biochim. Biophys. Acta,* 1954, v15, 194.

2. Koszalka, T. R., Falkenheim, R. N., Altman, K. I., *Biochim. Biophys. Acta,* 1957, v23, 647.

3. Schneider, W. C., Hogeboom, G. H., *J. Biol. Chem.,* 1952, v198, 155.

4. Koerner, J. F., Sinsheimer, R. L., *ibid.,* 1957, v228, 1039.

5. Schack, J., *ibid.,* 1957, v226, 573.

6. Hauck, P. B., Oser, B. L., Summerson, W. H., *Practical Physiological Chemistry,* The Blakiston Co., N. Y., 1947.

Received March 30, 1960. P.S.E.B.M., 1960, v104.

Protection of Mouse Bone Marrow by Inorganic Compounds During Freezing and Thawing. (25846)

PHAN THE TRAN* AND M. A BENDER (Introduced by C. C. Congdon)
Biology Division, Oak Ridge National Lab.,† Oak Ridge, Tenn.

It has long been known that glycerol protects mammalian cells against freezing and thawing damage(1). Lovelock showed that human red blood cells are protected against hemolysis not only by glycerol but also by a number of neutral organic solutes of low molecular weight(2). He attributed freezing damage to the increase in electrolyte concentration that occurs when pure ice is formed in and around the cells, and the protective action of organic solutes to reduction of electrolyte concentration in and around the cells because of the colligative properties of solutions. However, low concentrations of inorganic ions will protect bacteria against freezing and thawing damage(3). A similar protection, if it could be demonstrated for mammalian cells, would be contrary to Lovelock's hypothesis. We tested a number of inorganic compounds for ability to protect mouse bone marrow cells against freezing and thawing

* Visiting investigator on leave from Atomic Energy Office, Saigon, Viet Nam. Present address: Atomic Energy Office, Saigon, Viet Nam.

† Operated by Union Carbide Corp. for U. S. Atomic Energy Comm.

death. Mouse bone marrow cells have been preserved by the Polge-Smith-Parkes technic of slow-freezing in glycerol solutions and, after thawing, used successfully to promote recovery of lethally irradiated mice(4,5). It has been shown that recovery is the result of replacement of the host's damaged hematopoietic system by proliferation of the donor's cells(6). Our previous experiments showed that mouse bone marrow cells could also be successfully slow-frozen in presence of organic molecules other than glycerol. Many other polyalcohols and mono- and disaccharides are effective(7,8,9).

Materials and methods. The technical details of these experiments are identical to those described previously(7). Suspensions of femoral bone marrow from (C57BL X 101)F_1 donor mice were made in saline containing 3.5% polyvinylpyrrolidone (PVP)‡ and the compound to be tested. PVP, previously shown to be nonprotective, was used to prevent cell clumping. Samples of suspension (1 ml) were frozen in a bath whose temperature dropped at a rate of 1°C/min from +20° to −25°C(10). The tubes were then plunged into liquid nitrogen and kept at −196°C for 1 hour. Samples were thawed by agitation in a 37°C water bath, the cells were counted in a hemocytometer, and the suspension was diluted with saline to a concentration of 2 X 10^6 nucleated cells/ml. Lethally irradiated (900 r) isologous host mice were each given 0.5 ml of this suspension *via* the tail vein. Eosin-uptake measurements(11) were also made to determine the percentage of nonstaining (and presumably viable) cells. Seven simple inorganic molecules were tested using 8 different concentrations of each.

Results are given in Table I. The mice not given bone marrow all died, but 75% of control mice that had received 10^6 fresh bone marrow cells survived more than 30 days. Mice given marrow frozen in either physiological saline (0.154 M NaCl) or in Tyrode's solution did not survive. Of 7 neutral salts used, treatment with 6 (NaI, NaBr, $NaNO_3$, Na_2SO_4, $Na_2S_2O_3$ and NaSCN) gave fairly good 30-day survival at some concentrations, and even the seventh, $NaNO_2$, gave a few survivors. The eosin-uptake measurements seemed to be of little value in estimating recovery-promoting power of cells frozen in these solutions.

Discussion. The data in Table I suggest that there are 2 concentrations of each compound that give high protection. Thus, with NaSCN, survival is obtained between 0.003

TABLE I. Thirty-Day Survival of Lethally Irradiated Mice after Infusion of 10^6 Nucleated Bone Marrow Cells Frozen and Thawed in Solutions of Inorganic Salts.

Solution	Conc. (M) of salt in 3.5% PVP in saline	No. of mice	Eosin-negative cells (%)	30-day survival (%)
None		210		0
Fresh		288	84	75
Frozen in saline		81	3	0
Frozen in Tyrode's		58	5	0
Frozen in NaI	.003	18	11	6
	.006	"	11	6
	.013	"	19	0
	.025	20	35	50
	.05	"	23	35
	.1	"	3	10
	.2	23	2	0
	.4	20	0	0
Frozen in NaBr	.003	16	5	25
	.006	20	16	55
	.013	"	9	40
	.025	"	6	45
	.05	"	4	40
	.1	"	1	25
	.2	"	2	35
	.4	"	1	0
Frozen in $NaNO_3$	.003	"	19	40
	.006	"	14	65
	.013	"	12	95
	.025	"	13	45
	.05	"	17	45
	.1	"	9	15
	.2	22	3	59
	.4	20	0	0
Frozen in $NaNO_2$	.003	"	7	5
	.006	"	11	0
	.013	"	10	0
	.025	"	7	0
	.05	"	12	5
	.1	"	9	0
	.2	"	5	0
	.4	"	2	0
Frozen in Na_2SO_4	.003	"	6	60
	.006	"	2	0
	.013	"	1	50
	.025	"	1	5
	.05	"	7	15
	.1	"	5	0
	.2	"	2	5
	.4	"	0	0

‡ Vinisil, Abbott Labs., North Chicago, Ill.

TABLE I (continued).

Solution	Conc. (M) of salt in 3.5% PVP in saline	No. of mice	Eosin-negative cells (%)	30-day survival (%)
Frozen in NaSCN	.003	20	13	30
	.006	"	15	40
	.013	"	10	5
	.025	"	9	0
	.05	"	6	0
	.1	"	1	0
	.2	"	0	5
	.4	"	0	5
Frozen in $Na_2S_2O_3$	.003	"	2	15
	.006	"	4	0
	.013	"	3	30
	.025	"	3	25
	.05	"	5	20
	.1	"	6	35
	.2	"	16	5
	.4	"	2	0

and 0.013 M and between 0.2 and 0.4 M but not at intermediate concentrations. Although the data are not extensive enough to establish a bimodal concentration-protection curve for any one compound, the fact that all the compounds tested showed some effect seems significant. This phenomenon has been noted previously with protective organic compounds (7). Its significance, however, remains obscure.

A small amount of protective compound was injected into each mouse along with the test cells. The dose of compound varied from a minimum of 0.056 mmole/kg up to 12.8 mmole/kg. It seems extremely unlikely that this quantity by itself could have any protective effect on the mice.

It is difficult to explain the protection of mammalian cells against freezing and thawing damage by inorganic salts. It cannot, however, be attributable to a simple lowering of electrolyte concentration during freezing, as was suggested by Lovelock(2) for organic solutes. Neither can it be the result of vitrification of cellular fluid as suggested by Luyet (12). More quantitative physical data, preferably on individual cells, may help to explain the observed phenomena.

Summary. The ability of inorganic compounds to protect mouse bone marrow cells against freezing and thawing damage has been investigated, using a lethally irradiated isologous recipient as a functional assay of viability. NaI, NaBr, $NaNO_3$, $NaNO_2$, Na_2SO_4, NaSCN, and $Na_2S_2O_3$ were tested. All showed protective action against freezing and thawing. The effectiveness of different inorganic salts varied greatly. Each compound was effective only at certain concentrations.

1. Polge, C., Smith, A. U., Parkes, A. S., *Nature,* 1949, v164, 666.

2. Lovelock, J. E., *Biochem. J.,* 1954, v56, 265.

3. Lion, M. B., 1959, PhD Thesis, Hebrew Univ., Jerusalem.

4. Schwartz, I. R., Repplinger, E. F., Congdon, C. C., Tocantins, L. M., *J. Appl. Physiol.,* 1957, v11, 22.

5. Ferrebee, J. W., Billen, D., Urso, I. M., Lu, W. C., Thomas, E. D., Congdon, C. C., *Blood,* 1957, v12, 1096.

6. Congdon, C. C., 1959, in, *Progress in Hematology,* vII (ed. L. M. Tocantins), New York: Grune & Stratton, pp. 21-46.

7. Bender, M. A, Phan The Tran, Smith, L. H., *J. Appl. Physiol.,*

8. Phan The Tran, Bender M. A, *J. Appl. Physiol.,*

9. ———, *C.R. Soc. Biol. (Paris),*

10. Polge, C., Lovelock, J. E., *Vet. Rec.,* 1952, v64, 396.

11. Schrek, R., *Am. J. Cancer,* 1936, v28, 389.

12. Luyet, B. J., 1952, in *Freezing and Drying* (Ed., R. J. C. Harris), N. Y.: Hafner Publishing Co., pp. 77-98.

Received April 4, 1960. P.S.E.B.M., 1960, v104.

Distribution in Blood and Excretion of Zn^{65} in Man.* (25847)

FRANK A. GRAIG AND EDWARD SIEGEL (Introduced by T. Spaet)
Medical Physics Laboratory, Montefiore Hospital, N. Y. C.

In studying radiozinc localization in the prostate gland, it was desired to learn more about distribution and excretion of this radioisotope in man, since relevant literature(1-10) leaves many questions unanswered.

Materials and methods. 17 patients were given Zn^{65} chloride (specific activity greater than 75 mc/g) in intravenous doses, ranging from 50 μc to 100 μc. All patients but one suffered from various neoplastic diseases; none had serious impairment of liver or kidney function. Two of the group were receiving steroid therapy. Activities at various intervals up to 60 days of whole blood, plasma and red cells were determined. No studies of Zn^{65} concentration of leucocytes were attempted, since this has been extensively studied by others(9). Erythrocytes were hemolyzed with distilled water, and the supernatant fluid as well as the sediment, consisting of red cell ghosts (and leucocytes), were counted separately. Red cells were washed 3 times before assay; their sediment was washed until colorless. Plasma was dialyzed against normal saline. Urine and stool were collected to 2 weeks, as completely as possible. Daily urine samples less than 700 ml and 24 hr stool specimens weighing less than 100 g were excluded. Urine dialysis was performed against polyvinylpyrrolidone (PVP). Liquid samples were assayed in duplicate in a well-type scintillation counter. Stool activity was measured with a scintillation probe, positioned 15 cm from top of specimen container. Adequate counts were collected to assure an accuracy of greater than 10% for assay of stools and better than 5% for all other samples.

Results. 1. *Blood Zn^{65}.* Mean Zn^{65} concentrations (% dose/liter) of whole blood, erythrocytes, and plasma as a function of time are indicated in Fig. 1. The whole blood curve falls rapidly during the first 2 hrs (not indicated in figure) following injection, rises to its maximum on the eleventh day, and thereafter decreases slowly. Red cell concentration pattern is similar to that of whole

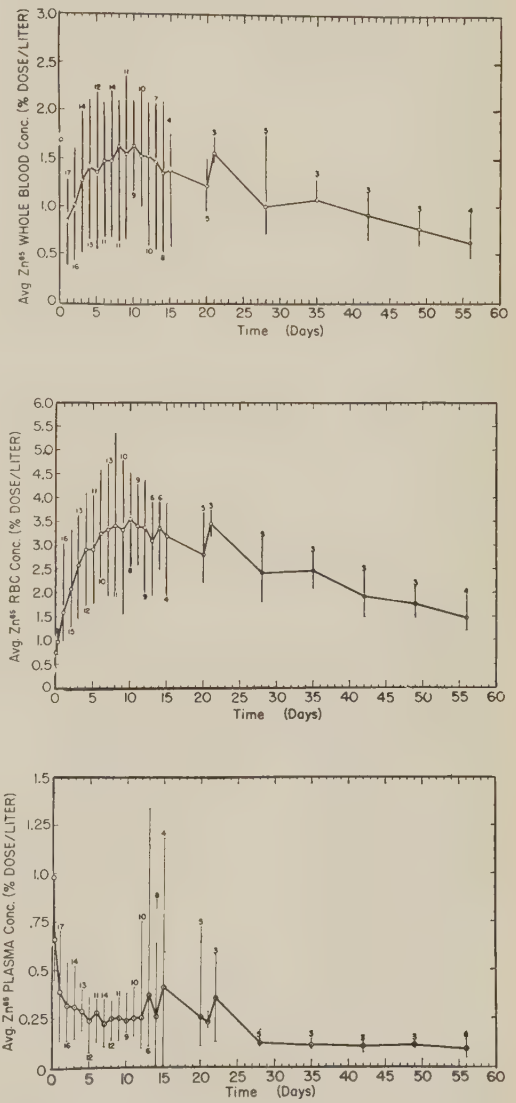


FIG. 1. Variation of Zn^{65} concentration of whole blood, RBC, and plasma with time. Individual points represent mean values of samples obtained from number of patients indicated at end of vertical lines extending from each point. Length of vertical line denotes range (max and min values).

* This investigation supported in part by grant from Nat. Cancer Inst., U.S.P.H.S.

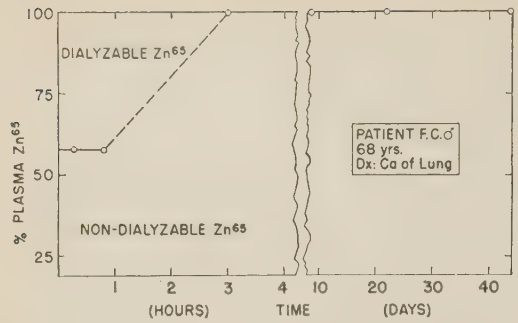


FIG. 2. Results of 24 hr dialysis of plasma samples obtained at various times following Zn^{65} administration, demonstrating binding of radiozinc to plasma proteins.

blood. Plasma curve falls continuously, although the range is considerable for several of the points. Semi-logarithmic plots of individual whole blood, red cell and plasma concentrations against time suggest that they can be resolved into exponential components. Zn^{65} erythrocyte concentration grows at a constant rapid rate for the first 4 days and at a second, slower rate thereafter until the eleventh day.

Plasma dialysis was performed on numerous samples obtained from 4 patients (Fig. 2) with essentially the same results. Up to one hour after injection, approximately one half of radiozinc freely traverses the membrane; by 3 hrs and until 40 days, virtually all is in a non-dialyzable form. Complete binding of radiozinc to plasma proteins at 3 hrs coincides with levelling of plasma concentration.

An example of hemolysis results, as studied in 6 cases, is given in Table I. Almost all radioactivity is found in the supernate. Dialysis of this solution demonstrates that Zn^{65} becomes protein-bound within the red cell.

TABLE I. Effect of Hemolysis on RBC Zn^{65} Concentration. (Patient H. N. ♂, 67 yr; bronchogenic Ca.)

Time from dose (days)	RBC Zn^{65} conc. (% dose/l)	Zn^{65} activity (% dose) after hemolysis	
		Supernate	Sediment
1	1.76	1.87	.056
2	2.14	2.27	.086
3	2.74	2.91	.065
4	3.21	3.11	.078
5	3.45	3.40	.096
6	3.16	3.46	.100
7	3.58	4.06	.068
9	3.80	3.22	.067
10	3.98	3.96	.071
11	3.95	3.95	.052
12	3.99	4.04	.061
23	3.35	3.43	.050

2. Urinary and stool Zn^{65}. Maximum urinary excretion rate (0.2 to 1.3% of dose per day) occurred during the first 2 days following injection. After the third day, daily urinary excretion remained quite constant, 0.2 to 0.6% of dose. Cumulative urinary and stool excretion for first and second weeks following Zn^{65} administration is given in Table II. During first week from 1.8 to 6.5% of dose appeared in the urine; by the end of second week, urinary output totalled between 3.3 and 11% of dose. Daily samples of urine from 2 patients were dialyzed and assayed with consistent results. Urinary Zn^{65} was almost entirely dialyzable (Table III), indicating that excretion of zinc occurs as a non-protein compound. Zn^{65} stool levels fluctuated widely from day to day. Total stool excretion of the first week ranged from 3.4 to 20% of dose. The patient with 20% fecal excretion had diarrhea throughout most of the collection period.

TABLE II. Cumulative Zn^{65} Urinary and Fecal Excretion (% Dose).

Sex & age	Diagnosis	Urine		Stool	
		1st wk	2nd wk	1st wk	2nd wk
♂ 52	Ca sigmoid	2.58	4.43		
♂ 76	" prostate	4.82	7.23		
♀ 51	" breast	2.13	3.30		
♂ 34	" lung	6.46	10.9	3.40	
♀ 70	Met. ca breast	1.79		8.87	
♀ 54	*Idem*	2.52		20.3	
♂ 65	Ca lung	4.40	6.50	4.56	7.60
♂ 49	Ca esophagus	2.11	3.80	3.43	7.42

TABLE III. Inorganic Fraction of Urinary Zn^{65}. (Patient G. S. ♂, 49 yr; Ca esophagus.)

Time from dose (days)	% dialyzable Zn^{65}	Time from dose (days)	% dialyzable Zn^{65}
1	97.1	8	98.9
2	95.3	9	98.5
3	95.3	10	95.5
4	95.9	11	99.2
5	96.8	12	98.7
6	97.4	13	99.3
7	97.7	14	99.3

Discussion. The slope of the whole blood graph reflects the rapid disappearance of isotope from plasma during the initial 24 hrs; thereafter it largely mirrors rate of uptake by the red cells. The plasma curve and red cell curve are similar to those published by Ross (7). Plasma Zn^{65} becomes non-dialyzable very shortly after injection, indicating that it is protein-bound. Since certain amino acids (11,12) and imidazole groups(13) generally combine avidly with zinc, it is not surprising that the radioisotope is bound rapidly to the great excess of plasma proteins.

The gradual increase of Zn^{65} red cell concentration tempts one to speculate that this signifies incorporation into young red cells. That the ascending phase of red cell concentration curve is apparently governed by 2 exponential functions, suggests as possibilities that radiozinc is picked up by erythrocytes only at a particular stage of maturation or that circulating red cells take up the isotope at a different rate than precursors in the bone marrow. Site of binding within the red cell is uncertain. While it was stated(14) that Zn^{65} does not exchange *in vitro* with the zinc of erythrocyte carbonic anhydrase, this has not been tested *in vivo*. It remains a possible binding site as does lactic dehydrogenase, another zinc metalloenzyme in the red cell(15). Hemoglobin itself, as indicated in a recent study(16), or some other protein without enzymatic activity may also be able to bind the radiozinc.

Our Zn^{65} urinary excretion data are in accord with published findings concerning stable zinc. They confirm the observation that urinary excretion of zinc is independent of urine volume(17) and that it occurs at a fairly constant rate after equilibration in plasma. It is apparent from the dialysis experiments that Zn^{65} is not excreted in a protein-bound form, a point about which there has been controversy.

Fecal excretion of Zn^{65} varied considerably in the patients studied. The cumulative stool values are consistent with those reported in dogs(1,4), in whom injected zinc was excreted largely through the gastrointestinal tract(18). Increased quantities of stainable zinc were found in small intestinal epithelium following its intravenous injection(19), and it was suggested that fecal excretion might depend on tissue saturation. Although it has been stated(20) that the average hospital diet contains sufficient amounts of zinc, it is possible that patients with chronic wasting illnesses have depleted body stores. The single case of very high fecal excretion in a patient with diarrhea suggests that besides poor intake, increased fecal losses might contribute to zinc depletion.

Summary. Distribution and concentration of Zn^{65} in whole blood, plasma and red cells were studied following intravenous infusion. Analyses of concentration curves suggest that they can be resolved into exponential components. Plasma Zn^{65} becomes protein-bound by 3 hrs following its injection and remains non-dialyzable at least up to 40 days. Red cell Zn^{65} becomes protein-bound and cannot be removed by dialysis from the hemoglobin solution. Excretion of Zn^{65} in the urine occurs presumably as an inorganic compound. Fecal excretion of Zn^{65} is variable and may depend on tissue saturation. The possibility exists that prolonged diarrhea may lead to zinc depletion.

We acknowledge excellent technical assistance of Joseph V. Marino and other members of Laboratory staff.

1. Montgomery, M. L., Sheline, G. E., Chaikoff, I. L., *J. Exp. Med.,* 1943, v78, 151.

2. Sheline, G. E., Chaikoff, I. L., Jones, H. B., Montgomery, M. L., *J. Biol. Chem.,* 1943, v149, 139.

3.———, *ibid.,* v147, 409.

4. Birnstengel, M., Stone, B., Richards, V., *Am. J. Physiol.,* 1957, v186, 377.

5. Vallee, B. L., Fluharty, R. G., *J. Clin. Invest.,* 1947, v26, 1199.

6. Vallee, B. L., Fluharty, R. G., Gibson, J. G., *Acta Contra Cancerum,* 1949, v6, 869.

7. Ross, J. F., Ebaugh, F. G., Jr., Talbot, T. R., Jr., *Trans. Assn. Am. Phys.,* 1958, v71, 322.

8. Vallee, B. L., *Physiol. Rev.,* 1959, v39, 443.

9. Vallee, B. L., Hoch, F. L., Hughes, W. L., *Arch. Biochem. et Biophys.,* 1954, v48, 347.

10. Daniel, O., Haddad, F., Prout, G., Whitmore, W. F., *Brit. J. Urol.,* 1956, v28, 271.

11. Albert, A., *Biochem. J.,* 1952, v50, 690.

12. Perkins, D. J., *ibid.,* 1953, v55, 649.

13. Edsall, J. T., Felsenfeld, G., Goodman, D. S., Gurd, F. R. N., *J. Am. Chem. Soc.,* 1954, v76, 3054.

14. Tupper, R., Watts, R. W. E., Wormall, A., *Biochem. J.,* 1952, v50, 429.

15. Altman, K. I., *Am. J. Med.,* 1959, v27, 936.

16. Sastry, K. S., Viswanathan, L., Ramaiah, A., Sarma, P. S., *Biochem. J.,* 1960, v74, 561.

17. Vikbladh, I., *Scand. J. Clin. Lab. Invest.,* Suppl. 2, 1951.

18. McCance, R. A., Widdowson, E. W., *Biochem. J.,* 1942, v36, 392.

19. Mager, M., McNary, F. M., Jr., Lionetti, F., *J. Histochem. Cytochem.,* 1953, v1, 493.

20. Vallee, B. L., Wacker, W. E. C., Bartholomay, A. F., Hoch, F. L., *New Eng. J. Med.,* 1957, v257, 1055.

Received April 5, 1960. P.S.E.B.M., 1960, v104.

Coenzyme Q_{10} and Succinate-Tetrazolium Reductase Activity of Proliferative Lesions of Liver.* (25848)

L. W. WATTENBERG AND J. A. GRONVALL

Dept. of Pathology, University of Minnesota School of Medicine, Minneapolis

Histochemical studies of the succinic dehydrogenase system using tetrazolium salt reduction technics have shown a decrease of succinate-tetrazolium reductase activity in a number of benign and malignant proliferative lesions(1,2,3). Recent work employing the tetrazolium salt, 2 - (p - iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT), has indicated that a quinone, presumably coenzyme Q_{10} (CoQ_{10}), serves as an intermediate electron transport agent in the succinate-INT reductase reaction and that content of quinone in normal tissues is below that required for maximum rate of activity of this reaction(4). In the present investigation quantitative histochemical determinations of succinate-INT reductase activity of sections of normal liver, regenerating liver and the Novikoff hepatoma have been carried out in presence of added CoQ_{10} and also in presence of a second quinone, 2-methyl-1,4-naphthoquinone (menadione). These experiments were performed to determine the individual roles of the primary dehydrogenase and quinone in decreases in reductase activity which have been observed. Parallel studies have also been carried out on a second reductase system, alpha-glycerophosphate-INT reductase, in which a quinone electron transport agent takes part in a manner similar to that of the succinate-INT reductase system(4).

Materials and methods. Quantitative procedures have been described(4). Frozen and dried sections 16 μ thick, with a dry weight of approximately 0.5 mg were incubated in a reaction mixture containing sodium succinate or sodium alpha-glycerophosphate, 0.05 M; NaCl, 0.11 M; KCl, 0.003 M; $MgSO_4$, 0.001 M; Na_2HPO_4, 0.03 M; acetone 5%; and INT, 0.4 mg/ml; pH was adjusted to 7.4. Sections were incubated at 37° rather than 28° as in the former investigation because of inclusion in this study of tissues with very low activity. At the higher temperature the enhancing effect of the quinones is relatively less compared to total activity of the section than at the lower temperature. An incubation period of 15 minutes was used for determination of succinate-INT reductase activity and 30 minutes for alpha-glycerophosphate-INT reductase activity. As in the earlier work, addition of CoQ_{10} is made by coating the compound on the coverslip and placing the tissue section in direct contact with the dried material. A considerably greater enhancement of enzyme activity has been found with this method than by placing the quinone directly into reaction mixture. For this procedure, 0.02 ml of a solution of CoQ_{10}, 1 mg/ml, in equal volumes of ethyl ether and acetone was placed on a 22 mm square coverslip and the solvent allowed to evaporate at room tempera-

* This investigation was supported by Field Investigation Grant of Nat. Cancer Inst., N.I.H., U.S.P.H.S., Bethesda, Md.

TABLE I. Effects of Quinones on Succinate-INT Reductase and Alpha-Glycerophosphate-INT Reductase Activities of Normal and Rapidly Proliferating Hepatic Cells.

		Quinone additions			Quinone additions	
		None	CoQ_{10}	Menadione	CoQ_{10}	Menadione
		Enzyme activity*			Increased enzyme activity	
Enzyme system	Type of hepatic tissue	Units†	Units†	Units†	%‡	%‡
Succinate-INT reductase	Normal	190 ± 13	283 ± 28	312 ± 26	49	64
	24 hr regenerating	127 ± 20	196 ± 20	283 ± 29	54	123
	48 " "	91 ± 3	159 ± 19	243 ± 19	75	167
	Hepatoma	54 ± 10	221 ± 14	143 ± 7	310	165
Alpha-glycerophosphate-INT reductase	Normal	25 ± 5	42 ± 11	65 ± 12	68	160
	24 hr regenerating	14 ± 3	17 ± 4	60 ± 17	21	329
	48 " "	15 ± 4	19 ± 4	61 ± 8	27	307
	Hepatoma	52 ± 6	133 ± 18	210 ± 34	156	304

* Mean ± stand. dev. Each value represents 8 animals.
† 1000 × △O.D. at 490 mμ/mg dry wt/min.
‡ 100 × (activity with quinone addition − activity without addition)/activity without addition.

ture. Menadione was added directly to the reaction mixture and was present at a concentration of 0.2 mg/ml. Female Sprague-Dawley rats 3 to 4 months of age obtained from the Holtzman Co. were used. In the hepatic regeneration experiments approximately two-thirds of the liver was excised(5). The Novikoff hepatoma was grown intraperitoneally as a solid tumor(6). Tumors 4 or 5 days following transplantation were used for the quantitative work.

Results. The results are summarized in Table I. Succinate-INT reductase activity of regenerating liver and the Novikoff hepatoma is considerably less than that of the normal liver. Succinate-INT reductase of normal liver and the proliferating lesions of the liver are not saturated with respect to quinone as evidenced by the fact that in these tissues, activity of this system is enhanced by addition of either CoQ_{10} or menadione to the reaction. In regenerating liver, degree of enhancement obtained with CoQ_{10} is slightly greater than in normal liver. Addition of menadione causes a considerably greater enhancement of reductase activity than that found in the normal liver, with the result that activity of regenerating liver approaches that of normal liver when both are incubated in presence of this quinone. In the Novikoff hepatoma, CoQ_{10} enhances succinic-INT reductase activity to a very marked degree so that addition of this quinone brings activity of the hepatoma close to that of normal liver incubated under the same conditions. Menadione is less effective in this regard.

The results obtained with alpha-glycerophosphate-INT reductase system in certain respects parallel those of succinate-INT reductase. Thus, in the regenerating liver, activities of both reductases are profoundly enhanced by menadione whereas CoQ_{10} is considerably less effective. An additional parallelism is found in the hepatoma. In this instance, activities of both of the reductases are markedly enhanced by CoQ_{10}. A major point of difference is that alpha-glycerophosphate-INT reductase activity of the hepatoma is greater than that of the normal liver, in sharp contrast to the situation existing with succinate-INT reductase.

Discussion. The frequent occurrence of low succinate-tetrazolium reductase activity in rapidly proliferating tissues suggests that a decrease in activity of this system is part of a metabolic alteration associated with rapid cellular proliferation in at least certain anatomic structures. The results of the present work indicate that in liver a considerable proportion of this decrease in activity is due to an alteration in the quinone component of the system. Recently reported data indicate that CoQ_{10} is an intermediate electron transport agent between succinic dehydrogenase and the cytochrome chain(7). Accordingly, the findings presented here suggest the possibility that in

proliferating cells one or more mechanisms may exist which result in succinic dehydrogenase and also alpha-glycerophosphate dehydrogenase becoming dissociated from CoQ_{10} and the cytochrome electron transport chain.

The nature of the alteration of quinone-dehydrogenase relationship which occurs in the regenerating liver and hepatoma appear to differ. In considering the pattern of response of the reductases of regenerating liver to the 2 quinones, account should be taken of the fact that regenerating liver is a sharply self-limited proliferative process occurring in an adult tissue. Accordingly, an attractive hypothesis for explaining the effects of the 2 quinones on this tissue is that the proliferative mechanism is related to a reversible dissociation of the 2 dehydrogenases from the electron transport system by virtue of some effect on CoQ_{10}. Under these conditions it might be more difficult for CoQ_{10} added *in vitro* to couple with the dehydrogenase than it would be for an unnatural quinone such as menadione. In the hepatoma this type of postulated reversible mechanism would presumably be lacking. The pronounced enhancing effect of CoQ_{10} on the 2 reductases of the hepatoma suggest that in this tissue there simply is a lack of available quinone which is most efficiently supplied *in vitro* by addition of the naturally occurring quinone.

Conclusion. Quantitative histochemical studies of sections of normal liver, regenerating liver and Novikoff hepatoma have shown that the succinate-INT and alpha-glycerophosphate-INT reductase systems of these tissues are not saturated with respect to quinone. Compared to normal liver, degree of unsaturation is considerably greater in regenerating liver and hepatoma and largely accounts for those low reductase activities which are observed in these 2 tissues. Response of sections of regenerating liver and hepatoma to CoQ_{10} and menadione differs. CoQ_{10} is very effective in hepatoma. In regenerating liver CoQ_{10} is relatively ineffective whereas enhancement of reductase activity brought about by menadione is profound.

1. Wattenberg, L. W., *Cancer Res.*, 1959, v19, 1118.
2. Ogawa, K., Zimmerman, H. M., *J. Histochem. Cytochem.*, 1959, v7, 342.
3. Monis, B., Nachlas, M. M., Seligman, A. M., *Cancer*, 1959, v12, 1238.
4. Wattenberg, L. W., *J. Histochem. Cytochem.*, 1960, v8.
5. Higgins, G. M., Anderson, R. M., *Arch. Path.*, 1931, v12, 186.
6. Novikoff, A. B., *Cancer Res.*, 1957, v17, 1010.
7. Green, D. E., Lester, R. L., *Fed. Proc.*, 1959, v18, 987.

Received April 6, 1960. P.S.E.B.M., 1960, v104.

Dietary Fats and Effects of Internal Radiation by P^{32}.* (25849)

CAMILLO ARTOM AND HUGH B. LOFLAND, JR.†
Dept. of Biochemistry, Bowman Gray School of Medicine, Wake Forest College, Winston-Salem, N. C.

Several groups of workers reported that addition of corn oil, cottonseed oil, or methyl linoleate to a fat-free diet significantly decreased mortality of mice(1) or rats(2-6) subjected to total body irradiation. On the other hand, our previous data indicated that mice injected with single midlethal dose of P^{32} exhibited greater susceptibility to the isotope when fat content of diet was raised from 5% to 30% level(7). We have now compared effects of P^{32} on mice fed a fat-free diet with those observed in mice fed diets with high content of either a saturated fat (coconut oil), or an unsaturated fat (corn oil). Mortality was lowest in groups on fat-free diet. However, mice fed corn oil showed a better survival than those on coconut oil diet.

* Work done under contract with U. S. Atomic Energy Comm.
† Technical assistance was given by C. Downs.

TABLE I. Composition of Experimental Diets (Values in g/100 g of Diet).

	Diet A	Diet B	Diet C
Casein	20	20	20
Dextrin	36	21	21
Sucrose	36	21	21
Hydrogenated coconut oil			30
Corn oil		30	

In addition, all diets contained: Salt mixture (U.S.P. XIV),* 4 g; vitamin diet fortification mixture,* 2 g; cellulose ("Alphacel"*), 2 g.

* Nutritional Biochemicals Corp., Cleveland, O.

The beneficial role of EFA‡ became more clearly apparent in experiments in which fat-free diets, supplemented with small amounts of coconut oil, corn oil, or linoleic acid, were used, and mice maintained on diets for 2 to 4 weeks before injection of P^{32}.

Methods. In each experiment, 120 to 160 male albino mice (Rockland Farm "all-purpose" strain) with average initial body weight of 20 g were distributed in groups of 8 or 9, and housed in metallic cages with raised screen floors. In each of Exp. 1 to 8, groups were maintained on fat-free diet (Diet A), or on a diet containing corn oil (Diet B), or coconut oil (Diet C) (Table I). In Exp. 9 to 11, all mice received fat-free diet plus one of following supplements: coconut oil, 3% (Diet A-1), corn oil, 3% (Diet A-2), or linoleic acid, 1.5% (Diet A-3). These supplements were added at the expense of dextrin. Animals were maintained on experimental diets for 3 to 15 days (Exp. 1-8), or 17 to 28 days (Exp. 9-11), then most mice were injected intraperitoneally with 0.2-0.3 ml of Ringer solution of phosphate containing P^{32} and continued on same diets for 6 more weeks. Amounts of P^{32} injected/g of mouse were constant in each experiment, but varied between 4.15 and 5 μc/g in different experiments. Control groups, maintained on experimental diets for same periods, were injected with Ringer solution only.

Results. As in previous investigations(7-10), the following criteria were used as indications of injurious effects of the isotope: a) time of 50% deaths; b) and c) % of survivors at 21st and 42nd day after injection; and d) average survival time, a 42-day survival being assigned to mice still alive when experiments were discontinued. In each experiment, data obtained on groups receiving P^{32} have been corrected for mortality of control groups maintained on same diet, but not injected with P^{32}. In most cases significance of the difference observed between weighted averages was appraised statistically by applying the *Chi*^{2} method to numbers of survivors and the *t* test(11) to survival times.

It is apparent (Tables II and III) that mice fed fat-free Diet A survived in greater numbers and for longer times than those fed Diets B and C. These results agree with our previous experiments(7) with diets containing a definite proportion of highly unsaturated fat (cod-liver oil) and varying amounts of fat with low iodine number ("Crisco").

On the other hand, the present results show

TABLE II. Survival of Mice Injected with P^{32} and Maintained on a Fat-Free Diet (Diet A), or on High-Fat Diets (Diets B and C).

	Diet A (fat-free)	Diet B (corn oil, 30%)	Diet C (coconut oil, 30%)
No. of mice inj. with P^{32}	216	326	348
Avg food consumption/mouse/day, g	3.1	3.3	2.9
" change in body wt (42 days),* g	+ .50	−1.09	−1.26
Time of 50% deaths, days	42	42	19
Survivors at 21 day, %	81	58	45
Survivors at 42 ", %	76	52	39
Avg survival time, days†	36 ± .8	33 ± .7	28 ± .7

Values are avg of 8 experiments, and adjusted on basis of values obtained from control groups not inj. with P^{32}. Mice were maintained on experimental diets for 3 to 15 days (avg, 7.8 days) before inj. of P^{32}. Doses of P^{32} inj. varied between 4.15 and 5 μc/g (avg, 4.34 μc/g).

* Survivors only.

† Values preceded by ± are stand. errors of means.

‡ EFA = Essential fatty acids.

TABLE III. Statistical Comparison of Survival of Mice Injected with P^{32} and Maintained on a Fat-Free Diet (Diet A), or on Diets Containing Corn Oil, 30% (Diet B), or Hydrogenated Coconut Oil, 30% (Diet C).

Diets compared	No. of mice inj. with P^{32}	No. of survivors 21 days Chi^2	21 days P*	42 days Chi^2	42 days P*	Avg survival time t†	P*
A *vs* C	542	83	<.01	40	<.01	7.6	<.01
A *vs* B	564	28	"	13	"	2.5	<.02
B *vs* C	674	10	"	15	"	5.2	<.01

* Probability for a chance occurrence.

† t, according to Fisher(11).

that the diet containing a high % of corn oil gave significantly better survival than the diet containing hydrogenated coconut oil. There were no marked differences in food consumption and body weight change, nor were other symptoms of EFA deficiency demonstrable.

The beneficial effects of EFA are further illustrated by results of Exp. 9-11 (Tables IV and V), in which small amounts of coconut oil, or corn oil, or linoleic acid were added to fat-free Diet A. In addition, prior to injection of the isotope, mice were maintained on diets for longer periods (2 to 4 weeks). Even under these conditions, typical signs of EFA deficiency were not seen. However, mice injected with P^{32} and maintained on EFA-free diet died sooner and in a larger proportion than those receiving small amounts of corn oil or linoleic acid. Except for the % of survivors at 42nd day, no significant differences were found in survival of animals receiving either corn oil or linoleic acid.

In conclusion, it appears that there is no actual discrepancy between our previous observation of higher mortality in mice injected with P^{32} and fed high-fat diets, and experiments of other investigators showing that addition of fats rich in EFA to a fat-free diet gives a significant protection against total body X-irradiation. Presumably suscepti-

TABLE IV. Survival of Mice Injected with P^{32} and Maintained on Diet A, Supplemented with Hydrogenated Coconut Oil, 3% (Diet A-1), Corn Oil, 3% (Diet A-2), or Linoleic Acid, 1.5% (Diet A-3).

	Diet A-1 (coconut oil, 3%)	Diet A-2 (corn oil, 3%)	Diet A-3 (linoleic acid, 1.5%)
No. of mice inj. with P^{32}	106	92	91
Avg food consumption/mouse/day, g	1.5	2.8	2.2
" change in body wt (42 days),* g	−.95	−.64	−.90
Time of 50% deaths, days	11	14	16
Survivors at 21 day, %	13	40	41
Survivors at 42 ", %	10	40	22
Avg survival time, days†	15.4 ± .9	25.3 ± .9	23.1 ± 1.2

Values are avg of 3 experiments, and adjusted on basis of values obtained from control groups, not inj. with P^{32}. Before inj. of P^{32}, mice were maintained on experimental diets 17 to 28 days (avg, 24 days). Doses of P^{32} inj. varied from 4.5 to 4.75 μc/g (avg, 4.63 μc/g).

* Survivors only.

† Values preceded by ± are stand. errors of means.

TABLE V. Statistical Comparison of Survival of Mice Injected with P^{32} and Maintained on Diet A, Supplemented with Hydrogenated Coconut Oil, 3% (Diet A-1), Corn Oil, 3% (Diet A-2), or Linoleic Acid, 1.5% (Diet A-3).

Diets compared	No. of mice inj. with P^{32}	No. of survivors 21 days Chi^2	21 days P*	42 days Chi^2	42 days P*	Avg survival time t†	P*
A-1 *vs* A-2	198	18	<.01	25	<.01	7.5	<.01
A-1 *vs* A-3	197	20	"	6	<.05	5.2	"
A-2 *vs* A-3	183	1	>.05	6	"	1.4	>.05

* Probability for a chance occurrence.

† t, according to Fisher(11).

bility of animals toward radiation damage is affected both by level of dietary fat and its content in EFA. As long as a severe deficiency of EFA is not fully developed, mice injected with P^{32} and fed a fat-free diet exhibit better survival than mice fed diets containing high levels of fats, saturated or unsaturated. At present, this adverse effect of fat-rich diets is difficult to explain. It is conceivable that radiation damage may be more severe (or less easily reversible) in animals whose tissues are being progressively depleted of EFA.

Summary. Mice were maintained on various experimental diets and injected with single dose of radioactive phosphate (4-5 μc/g). Higher % of survivors and longer survival time were observed in animals on fat-free diet as compared with those fed diets containing 30% corn oil, or 30% hydrogenated coconut oil. However, with these high-fat diets, as well as with diets containing only minimal amounts of fats, a better survival was demonstrable when highly unsaturated fatty acids were present. It appears that, provided that definite amounts of these fatty acids are included, a low-fat diet should be beneficial in alleviating effects of internal radiation by P^{32}.

1. Decker, A. B., Fillerup, D. L., Mead, J. F., *J. Nutrition,* 1950, v41, 507.

2. Cheng, A. L. S., Kryder, G. D., Berquist, L., Deuel, H. J., Jr., *ibid.,* 1952, v48, 161.

3. Deuel, H. J., Jr., Cheng, A. L. S., Kryder, G. D., Bingemann, M. E., *Science,* 1953, v117, 254.

4. Cheng, A. L. S., Ryan, M., Alfin-Slater, R. B., Deuel, H. J., Jr., *J. Nutrition,* 1954, v52, 637.

5. Cheng, A. L. S., Alfin-Slater, R. B., Deuel, H. J., Jr., *ibid.,* 1954, v54, 201.

6. Cheng, A. L. S., Graham, T. M., Alfin-Slater, R. B., Deuel, H. J., Jr., *ibid.,* 1955, v55, 647.

7. Cornatzer, W. E., Harrell, G. T., Jr., Cayer, D., Artom, C., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 492.

8. Cornatzer, W. E., Artom, C., Harrell, G. T., Jr., *ibid.,* 1951, v76, 522.

9. Artom, C., Cornatzer, W. E., Harrell, G. T., Jr., *ibid.,* 1952, v79, 494.

10. Artom, C., *ibid.,* 1954, v86, 162.

11. Fisher, R. A., *Statistical Methods for Research Workers,* London, 1936, 6th Edition.

Received April 7, 1960. P.S.E.B.M., 1960, v104.

Pineal-Like Effects of Central Nervous System Tissue Extracts.* (25850)

MARK D. ALTSCHULE AND JOSEPH N. GIANCOLA
Lab. of Clinical Physiology, McLean Hospital, Waverley, Mass.

Ranzenhofer *et al.*(1) showed that chicken spinal cord contains something that retards gonadal and comb growth. Retardation of gonadal growth is one of the properties of certain pineal-tissue extracts(2,3). Another effect of such pineal extracts is to elevate blood glutathione and eosinophil counts in schizophrenic patients(2,4). It was therefore decided to study effects of steer-brain extracts on these measurements.

Material and methods. Patients used were all schizophrenic and had been hospitalized for at least a decade. Measurements of blood glutathione level, and of eosinophil count were made as previously described(4). Extracts used were as follows: (a) in 4 patients, extracts of steer-brain "septal" tissue† made by Heath's modification(5) of technic for making pineal extract reported elsewhere(4); (b) in 2 patients extracts of whole steer brain (excluding the pineal)‡ made according to method previously described for pineal extract(4).

Observations. In 4 patients given "septal" extract the amount of extract given daily was derived from 42.5, 85 or 170 g of tissue/day. Dose of extract equivalent to 42.5 g/day caused no significant changes (Case B; Case K); dose of extract equivalent to 85 g of septal tissue/day caused changes in blood gluta-

* Aided by grants from Commonwealth Fund and Wilson Labs.

† Supplied by Upjohn Co.

‡ Supplied by Wilson Lab.

thione level and eosinophil count equivalent to or less than that produced by 5 g of pineal tissue (Cases K, M, W); dose of extract equivalent to 170 g/day caused greater changes than the dose equivalent to 85 g in the same patient (Case W).

Giving whole brain extract (excluding pineal) caused changes in blood glutathione level

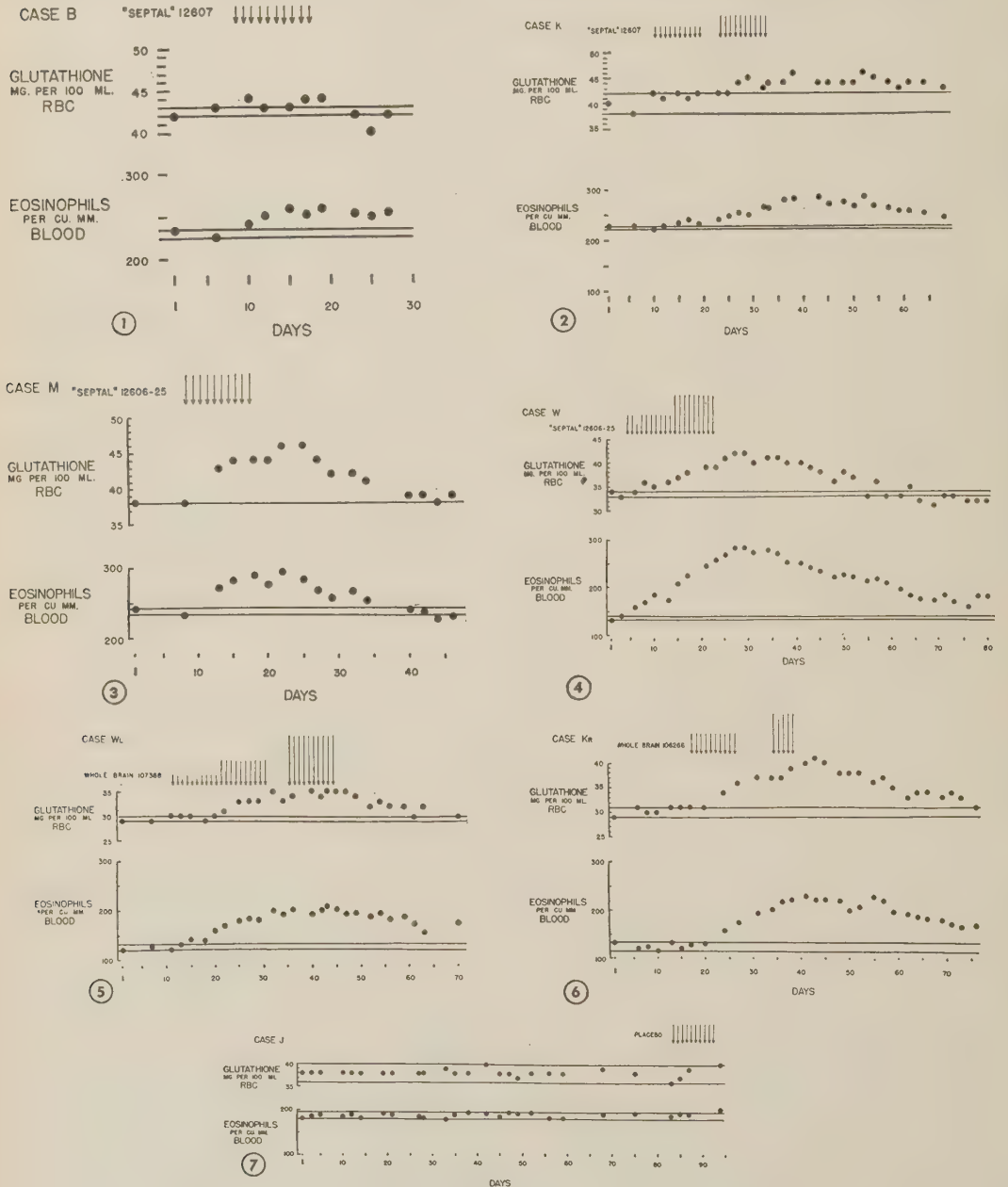


FIG. 1. Case B. Effects of extract derived from 42.5 g.
FIG. 2. Case K. *Idem* 42.5 and from 85 g.
FIG. 3. Case M. " 85 g.
FIG. 4. Case W. " 85 and from 170 g.
FIG. 5. Case Wl. " 10, 20 and 40 g.
FIG. 6. Case Kr. " 20 and 40 g.
FIG. 7. Case J. Effects of placebo.

and eosinophil count varying with dose used. A dose equivalent to 10 g of brain tissue caused no change (Case Wl). A dose equivalent to 20 g caused small rises in blood glutathione and eosinophil levels (Cases Wl and Kr); doubling the dose caused further rises in these measurements (Cases Wl and Kr).

In all experiments, measurements returned to control level some weeks after cessation of injections. Placebo injections had no effect (Case J). Placebos in this and other cases were extracts made either from beef ovary, liver, or skeletal or cardiac muscle.

Discussion. Our data showed that brain tissue contains a substance that causes biochemical changes in schizophrenic patients identical with those produced by certain pineal extracts. As far as can be judged from these crude experiments, concentration of this material in brain is about a fifth to a tenth of that found in the pineal gland.

No such activity was found in extracts of beef liver, ovary or skeletal or cardiac muscle.

The question whether brain manufactures this material, or whether it is distributed through the brain from the pineal gland cannot be answered. However Holmgren has shown that secretory granules leave the pineal gland and migrate to the third ventricle. If secretory material entered the spinal fluid in this fashion it could readily be distributed through the entire central nervous system. In this connection it is interesting that melatonin, another product of the pineal gland, is also widely distributed in nervous tissue(6).

Summary and conclusions. 1) Extracts from whole steer-brain (excluding pineal gland) cause the same changes in schizophrenic patients as do pineal extracts made the same way. Concentration of active material in brain tissue is very small. Substances which originate in the pineal gland appear to be widely distributed in the central nervous system. 2) Beef ovary, liver and skeletal and cardiac muscle contained no active material.

1. Ranzenhofer, E. R., Lipton, M. M., Steigman, A. J., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 280.

2. Kitay, J. I., Altschule, M. D., *The Pineal Gland.* Cambridge, Mass., Harvard Univ. Press. 1954, p32.

3. Wurtman, R. J., Altschule, M. D., Holmgren, U., *Am. J. Physiol.,* 1959, v197, 108.

4. Altschule, M. D., *New Eng. J. Med.,* 1957, v257, 919.

5. Heath, R. G., Leach, B. E., Byers, L. W., Martens, S., Feigley, C. A., *Am. J. Psychiat.,* 1958, v114, 683.

6. Lerner, A. B., Case, J. D., Mori, W., Wright, M. R., *Nature,* 1959, v183, 1821.

Received April 12, 1960. P.S.E.B.M., 1960, v104.

Action of Flavonoid Metabolites on Pituitary-Adrenal Axis.* (25851)

M. S. MASRI, C. W. MURRAY AND F. DEEDS
Western Regional Research Laboratory,† Albany, Calif.

We showed that certain flavonoids accelerate thymus involution through action on pituitary-adrenal axis, while others lack this effect(1,2). This comparison permitted some conclusions relating this action to structural configuration. Metabolic studies with several flavonoids showed they are readily absorbed and degraded to a variety of phenolic acids (3-9). The effect of phenolic acid metabolites of certain flavonoids and compounds related to these metabolites on thymus involution is reported here.

Methods. Female albino rats, 22-26 days old were fed for 12-14 days a semi-synthetic diet previously described(2) with or without addition of a phenolic acid (1%). They were

* Authors are indebted to Drs. Robert H. Wilson for reviewing statistical treatment and Albert N. Booth for interest in the work, and to Paulette Henderson for technical assistance.

† A laboratory of Western Utilization Research and Development Division, Agric. Research Service, U. S. Dept. of Agric.

TABLE I. Effect of Phenolic Acids on Thymus Involution in Normal Female Rats.

Acid fed	No. of rats	Final age, days	Mean final body wt, g	Mean thymus wt, % of body wt		
				Mean ± S.E.	Diff. ± S.E.	P*
None, basal alone	61	38	84.5	.354 ± .005		
3,4-Dihydroxybenzoic (protocatechuic)	11	41	82.5	.299 ± .007	.055 ± .009	<.001
2,4-Dihydroxybenzoic	10	35	77.1	.341 ± .013	.013 ± .014	
m-Hydroxybenzoic	9	38	81.7	.384 ± .028	−.030 ± .028	
p-Hydroxybenzoic	10	41	74.5	.335 ± .010	.019 ± .011	
3-Methoxy-4-hydroxybenzoic (vanillic)	9	38	76.6	.326 ± .050	.028 ± .050	
3,4-Dihydroxyphenylacetic	10	39	80.2	.318 ± .014	.036 ± .015	<.02
m-Hydroxyphenylacetic	10	35	76.0	.336 ± .013	.018 ± .014	
3,4-Dihydroxycinnamic (caffeic)	10	37	80.4	.320 ± .011	.034 ± .012	<.01
3-Methoxy-4-hydroxycinnamic (ferulic)	9	38	83.8	.351 ± .010	.003 ± .011	
m-Hydroxyphenylpropionic	10	35	69.2	.367 ± .018	−.013 ± .019	
p-Hydroxyphenylpropionic	10	35	68.6	.339 ± .012	.015 ± .013	

* P = Probability that such a difference would occur by chance. Only significant values of P are indicated.

sacrificed thereafter and body and thymus weights obtained. Body organs were examined for gross changes. Adrenalectomy (bilateral) was performed on 27-day-old females; these were immediately maintained on test diets and saline postoperatively for 12-14 days before sacrifice. Hypophysectomized females were 26 days old when pituitaries were removed; they were then put on test diets and sacrificed 2 weeks later. 3, 4-Dihydroxyphenylacetic and m-hydroxyphenylacetic acids were obtained by demethylation of corresponding methyl ethers. The following derivatives of cinnamic acid were prepared from corresponding benzaldehydes by condensation with malonic acid(10): m-hydroxycinnamic (m-coumaric), p-hydroxycinnamic (p-coumaric), 3,4-dihydroxycinnamic (caffeic), and 3-methoxy-4-hydroxycinnamic (ferulic) acids. Derivatives of phenylpropionic acid were prepared from corresponding cinnamic acids by hydrogenation using palladium over charcoal. Oxidation of vanillin with fresh silver oxide gave 3-methoxy-4-hydroxybenzoic (vanillic) acid(11). The following were purchased: m- and p-hydroxybenzoic and 3,4-dihydroxybenzoic (protocatechuic) acids.

Results. Effect on thymus involution of several phenolic acid metabolites of flavonoids or compounds related to these metabolites is shown in Table I. Average body weights and ages of different groups at time of sacrifice are also indicated. Accelerated involution is evident in groups receiving catecholic acids: 3,4-dihydroxyphenylacetic, caffeic and protocatechuic. Gross organ examination and growth rate of groups receiving these acids showed no changes from control group. Other acids in Table did not accelerate involution. Table II shows that either adrenalectomy or hypophysectomy abolished thymolytic response to 3,4-dihydroxyphenylacetic acid. Protocatechuic and caffeic acids were not tested in hypophysectomized or adrenalectomized rats.

Discussion. Our findings shed light on whether the action of flavonoids on the pituitary-adrenal axis is due to intact flavonoids or their metabolites or both. The flavonoids quercetin, dihydroquercetin, eriodictyol and luteolin all possess orthodihydroxyphenyl (catecholic) groupings and had thymolytic action(1,2); of these, only quercetin and dihydroquercetin yield a catecholic metabolite in the rat, namely, 3,4-dihydroxyphenylacetic acid(3,4,7,8). This acid accelerated thymus involution (Table I), through the pituitary-adrenal axis (Table II). Major metabolites of eriodictyol(5) and luteolin‡ in the rat, on

‡ Unpublished observations from this Laboratory.

TABLE II. Thymus Gland Weights of Adrenalectomized or Hypophysectomized Female Rats Fed Control Diet or Diet Containing 3,4-Dihydroxyphenylacetic Acid.

	Control diet			Diet containing 3,4-dihydroxyphenylacetic		
	No. of rats	Mean final body wt, g	Thymus, % of body wt	No. of rats	Mean final body wt, g	Thymus, % of body wt
Adrenalectomized	10	64.9	.460 ± .030	12	59.8	.474 ± .026
Hypophysectomized	9	49.6	.354 ± .014	8	51.1	.348 ± .009

the other hand, are m-hydroxyphenylpropionic and m-coumaric acids but no catecholic acids. m - Hydroxyphenylpropionic acid (which readily forms m-coumaric acid in the body) did not accelerate involution (Table I). Thus, it may be inferred that this action of flavonoids is due in part at least to the intact molecule and, in certain instances, may also be due to the metabolites they yield.

The flavonoids hesperetin, naringenin, and morin, which have no catecholic groups do not cause thymus involution(2) and produce phenolic metabolites(5,6,9), which also fail to cause thymus involution (Table I).

Certain non-catecholic phenolic acids such as salicylic, gentisic and γ-resorcylic markedly affect level of circulating corticosteroids(12, 13). Whether flavonoids possessing radicals of these acids (constituting the prime ring of flavonoid) also stimulate adrenal function merits consideration.

Summary. (1) Several phenolic acid metabolites of flavonoids and compounds related to these metabolites were tested for thymolytic action by feeding in the diet to young normal rats. (2) Catecholic acids 3,4-dihydroxyphenylacetic, protocatechuic and caffeic, accelerated thymus involution. Several other phenolic acids did not. (3) 3,4-Dihydroxyphenylacetic acid did not accelerate thymus involution in either hypophysectomized or adrenalectomized rats. (4) Whether the action of flavonoids on thymus involution is due to intact flavonoids or their metabolites is discussed.

1. Masri, M. S., DeEds, F., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 707.

2. Masri, M. S., Murray, C. W., *ibid.*, 1959, v101, 818.

3. Murray, C. W., Booth, A. N., DeEds, F., Jones, F. T., *J. Am. Pharm. Assn., Sci. Ed.*, 1954, v43, 361.

4. Booth, A. N., Murray, C. W., Jones, F. T., DeEds, F., *J. Biol. Chem.*, 1956, v223, 251.

5. Booth, A. N., Jones, F. T., DeEds, F., *J. Biol. Chem.*, 1958, v230, 661.

6. ———, *ibid.*, 1958, v233, 280.

7. Booth, A. N., DeEds, F., *J. Am. Pharm. Assn., Sci. Ed.*, 1958, v47, 183.

8. Masri, M. S., Booth, A. N., DeEds, F., *Arch. Biochem. and Biophys.*, 1959, v85, 284.

9. Douglass, C. D., *Federation Proc.*, 1958, v17, 213.

10. Vorsatz, F., *J. für Praktische Chemie,* 1936, v145, 265.

11. Pearl, I. A., *Organic Syntheses,* 1950, v30, 101.

12. Done, A. K., Ely, R. S., Kelley, V. C., PROC. SOC. EXP. BIOL. AND MED., 1956, v93, 294.

13. ———, *Metabolism,* 1958, v7, 52.

Received April 14, 1960. P.S.E.B.M., 1960, v104.

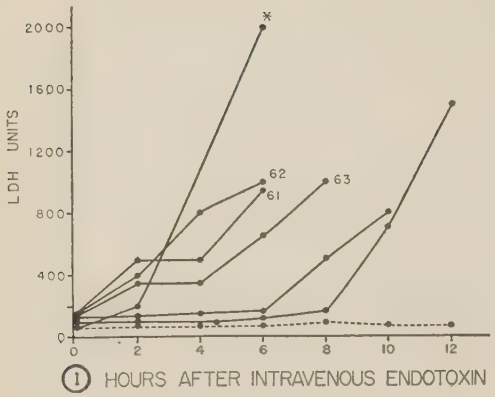
Serum Lactic Dehydrogenase Activity in Experimental Endotoxic Shock.* (25852)

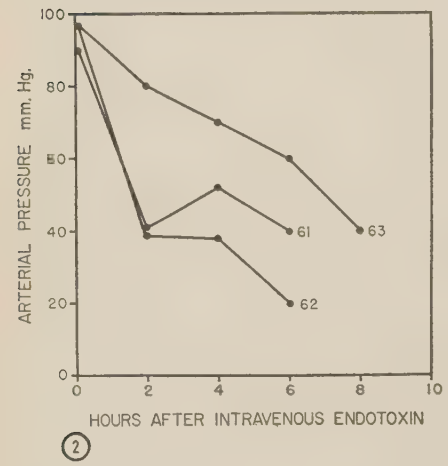
ELLIOT S. VESELL, CARLO F. P. PALMERIO AND EDWARD D. FRANK
(Introduced by J. Fine)
Dept. of Surgery, Harvard Medical School and Beth Israel Hospital, Boston, Mass.

Elevations of plasma lactic dehydrogenase (LDH) activity in experimental hemorrhagic shock have been described(1). This paper demonstrates elevations of serum LDH activity after endotoxin administration.

Materials and methods. One MLD_{100} of

* Aided by grant from Nat. Heart Inst., Bethesda, Md. and Office of Surgeon General, U. S. Army.





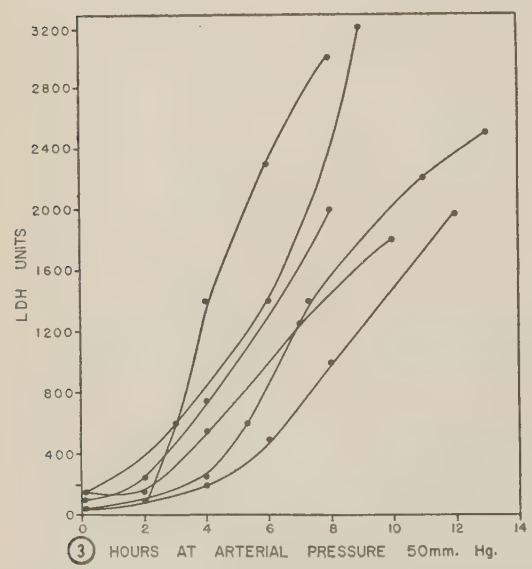


FIG. 1. Solid lines show serum LDH activity in 6 rabbits given one MLD_{100} of *Escherichia coli* endotoxin at time 0. Curve marked with asterisk reached 4000 units at 8 hr. Dotted line represents LDH activity in control rabbit given saline.

FIG. 2. Arterial pressures of rabbits 61, 62 and 63 following toxin administration.

FIG. 3. Plasma LDH activity of 6 rabbits during sustained hemorrhagic hypotension.

Escherichia coli endotoxin (Difco) was administered intravenously to adult albino rabbits (average weight 2 kg). Femoral artery pressure was obtained at intervals thereafter. In another group of similar rabbits hemorrhagic hypotension was produced by the technic described elsewhere(2). Arterial blood samples† were drawn before endotoxin administration or hemorrhagic hypotension and at intervals thereafter and the serum was collected at once, kept at 4°C for less than 24 hours, and then assayed spectrophotometrically for LDH activity(3).

Results. The serum LDH activity of 6 rabbits after endotoxin administration showed elevations to 800 to 4000 units, 6 to 50 times the initial value (Fig. 1). In 3 of these rabbits initial arterial pressures were 95, 95, and 90 mm Hg respectively before endotoxin administration. They began to fall 2 hours later and were 40, 40 and 20 mm Hg before death (Fig. 2). Death occurred between 6 and 18 hours after endotoxin was injected. Rabbits given saline intravenously showed no rise in serum LDH activity (Fig. 1).

Six rabbits subjected to hemorrhagic hypotension revealed plasma LDH elevations reaching 1800 to 3200 units (Fig. 3). Death occurred between 8 and 12 hours after bleeding was initiated.

Discussion. These experiments reveal progressive elevations of serum LDH activity in rabbits after endotoxin administration. Curves of the LDH activities of the 6 rabbits given endotoxin (Fig. 1) resemble the curves of the 6 rabbits in hemorrhagic hypotension (Fig. 3). This is in accord with other observations from this laboratory showing similarities between endotoxic and hemorrhagic shock(4). The mechanism of serum LDH alterations in disease states has not been fully established (5). Recent data suggest that elevations are due to release of enzyme from damaged tissues(1,6).

† No hemolysis was present.

Summary. 1. Marked elevations of serum LDH activity are described in rabbits given *Escherichia coli* endotoxin intravenously. Elevations reached 6 to 50 times the initial values. 2. Alterations in LDH activity of rabbits in endotoxic shock resemble those seen in hemorrhagic shock.

1. Vesell, E. S., Feldman, M. P., Frank, E. D., Proc. Soc. Exp. Biol. and Med., 1959, v101, 644.

2. Fine, J., Frank, H. A., Schweinburg, F. B., Jacob, S., Gordon, T., *Ann. N. Y. Acad. Sci.,* 1952, v55, 429.

3. Wróblewski, F., LaDue, J. S., Proc. Soc. Exp. Biol. and Med., 1955, v90, 210.

4. Fine, J., Rutenburg, S., Schweinburg, F. B., *J. Exp. Med.,* 1959, v110, 547.

5. Snodgrass, P. J., Wacker, W. E. C., Eppinger, E. C., Vallee, B. L., *N. Engl. J. Med.,* 1959, v261, 1259.

6. Strandjord, P. E., Thomas, K. E., White, L. P., *J. Clin. Invest.,* 1959, v38, 2111.

Received April 19, 1960. P.S.E.B.M., 1960, v104.

6-Aminopenicillanic Acid in Urine after Oral Administration of Penicillins. (25853)

Arthur R. English, H. T. Huang and B. A. Sobin

Research Labs., Chas. Pfizer and Co., Maywood, N. J., and Groton, Conn.

Sakaguchi and Murao(1) postulated existence of a hydrolytic enzyme capable of cleaving the acyl side-chain from the penicillin molecule. Batchelor *et al.*(2) isolated the actual penicillin moiety, 6-aminopenicillanic acid (6-APA) that results from such cleavage, as a fermentation product of *Penicillium chrysogenum.* Studies to determine whether or not 6-APA could be detected in urine of animal species after oral administration of Penicillin G and several other known penicillins are reported here.

Results. Penicillin G was administered orally at 500 mg/kg to mice housed in metabolism cages. Urine samples (feces free) were collected, pooled, and presence or absence of 6-APA determined by chromatographic procedure described by Batchelor *et al.*(2). The fact that 6-APA *per se* could be absorbed orally and appear in urine was shown by administration of this compound at 50 mg/kg to mice, and its subsequent detection in urine at 2, 6 and 24 hours.

The hydrolytic cleavage of acyl side-chain of Penicillin G *in vivo* was readily indicated by detection of 6-APA in urine of animals after oral administration of the intact antibiotic. 6-APA was detected within ½ hour and at 2, 6, and 24 hours in urine of mouse after oral or intravenous administration of Penicillin G (Table I). Similar results were found after oral administration of Penicillin G to rats, cats, rabbits, dogs,

TABLE I. Appearance of 6-Aminopenicillanic Acid in Urine of Animals after Administration of Various Penicillins.

Penicillins and dosage in mg/kg	Species	Route of admin.	Presence of 6-aminopenicillanic acid in urine*
6-Aminopenicillanic acid—50	Mouse	PO	Positive
Penicillin			
G—50, 500	Mouse	PO	Positive
V—500	"	"	"
V— "	"	IV	" †
G— "	Rat	PO	"
Dihydro F penicillin—1000	"	"	"
Penicillin			
K—1000	"	"	"
X— "	"	"	"
G— "	Dog	"	"
V— "	"	"	"
G— "	Cat	"	" ‡
G— "	Rabbit	"	"
V— "	"	"	"
G—200,000 units	Man	"	" §
G—500, 1000	Guinea pig	"	Negative
V— "	"	"	"

* Control urines from representative species were always negative. Sample times were at 2, 6, and 24 hr.

† Sample times at ½, 2½, and 3 hr.

‡ " " " 4 hr.

§ 24 hr samples.

TABLE II. Failure of Tissue Macerates to Hydrolyze Penicillins to 6-APA.

Species	Tissue or organ macerate	Conversion of penicillin G to 6-aminopenicillanic acid
Mouse	Kidney	Negative
Rat	Spleen	"
Rabbit	Liver	"
	Lung	"
	Brain	"
Mouse	Intestinal contents	Positive

and man. However, 6-APA was not detected in urine of guinea pig after repeated trials and dosages. Phenoxymethyl penicillin and other known penicillins used instead of Penicillin G gave similar results. (Table I).

Penicillin G was also detected in urines by chromatographic technics after oral administration of this antibiotic. Indications of possible conversion of Penicillin G to parahydroxy penicillin G (Penicillin X) were observed in the chromatographic systems. After oral administration of Penicillin V, presence of parahydroxy penicillin V was indicated by the chromatographic systems. Further experiments are in progress concerning this aspect.

Quantitative conversion in the mouse of administered dose of Penicillin G to 6-APA as appears in urine is quite inefficient, approximately 0.1-1% in these experiments.

In a concomitant series of experiments, enzymatic capacity of various macerated tissues from animal species to hydrolyze Penicillin G to 6-APA was determined. Animals were sacrificed, the individual organs removed, and macerated in aseptic manner in tissue grinder. Penicillin G (200 μg/ml; 1 mg/ml) was added to macerates and incubated 2-4 hours at 37°C in water bath. Enzymatic conversion of Penicillin G to 6-APA failed to occur with a variety of macerates from different animals under our conditions (Table II). When Penicillin G was added to a water suspension of intestinal tract contents of the mouse, 6-APA was detected. It appears that tissue macerates of the mouse, rabbit, guinea pig, and rat did not contain penicillin acylase activity. It might be postulated that presence of 6-APA in urine results from hydrolytic conversion of Penicillin G to 6-APA by the microbial flora in the intestine and subsequent absorption and excretion of 6-APA.

Possibly *in vivo* failure of penicillin conversion to 6-APA in the guinea pig is a reflection of an unusual microbial intestinal flora in this species, *i.e., Escherichia coli* is either not found at all in healthy guinea pigs, or found in rare cases and in minimal numbers. The predominant flora of guinea pig intestine are Gram positive, *Bacillus, Lactobacillus,* etc. (3,4).

Relevant to appearance of 6-APA in urine of the mouse after intravenous administration of Penicillin G, one might postulate appearance of Penicillin G in the intestine *via* transport in bile, subsequent penicillin acylase activity of intestinal flora, and absorption of 6-APA from intestine and excretion in urine.

Presence of penicillin acylase activity in a number of different microorganisms, such as genera *Escherichia, Hemophilus, Alcaligenes, Micrococcus, Pseudomonas,* and *Nocardia* is described by Huang *et al.*(5).

As noted elsewhere, flora of the guinea pig are predominantly *Bacillus* and *Lactobacillus.* These genera failed to convert Penicillin G to 6-APA.

Summary. 1) After oral and intravenous administration of Penicillin G and other known penicillins to mice, and oral administration to rats, dogs, rabbits, and man, 6-APA was detected in urine. After oral administration of penicillin to guinea pigs 6-APA was not detected in urine. 2) Tissue macerates of these animals failed to convert Penicillin G to 6-APA. 3) Probably 6-APA in urine is the result of microbial hydrolytic conversion of penicillin in the intestine and subsequent absorption and excretion.

1. Sakaguchi, K., Murao, S. J., *Agric. Chem. Soc. Japan,* 1950, v22, 411.

2. Batchelor, F. R., Doyle, F. P., Nayler, T. H. C., Rolinson, G. N., *Nature,* 1959, v183, 257.

3. Toshkov, A., Sheikova, G., Shipolini, R., *J. Microbiol., Epidemiol. Immunobiol.,* 1958, v29, 1056.

4. Fischer, G. W., *Zbl. Bakt. Abt. Orig.,* 1955, v164, 1230.

5. Huang, H. T., English, A. R., Seto, T. A., Shull, G. M., Sobin, B. A., *J. Am. Chem. Soc.,* 1960.

Received April 21, 1960. P.S.E.B.M., 1960, v104.

Variations in Reduction of Tetrazolium Salts by Dehydrogenase Systems.*† (25854)

Marvin M. Nachlas, Shankar S. Karmarkar and Arnold M. Seligman
Depts. of Surgery, Sinai Hospital of Baltimore, and Johns Hopkins University School of Medicine, Baltimore, Md.

For assay of dehydrogenase activity in homogenates the monotetrazolium salt, 2-*p*-nitrophenyl - 3 - *p* - iodophenyl - 5 - phenyl tetrazolium chloride (INT), has been used most extensively(1,2). For histochemical demonstration of dehydrogenase activity in frozen sections the ditetrazolium salts, 2, 2′,5,5′-tetraphenyl - 3,3′ - (3,3′ - dimethoxy - 4,4′ - biphenylene) ditetrazolium chloride (BT), 2, 2′,5,5′ - tetraphenyl - 3,3′ - (4,4′ - biphenylene) ditetrazolium chloride (NT), and more recently 2,2′ - di - *p* - nitrophenyl - 5,5′ - diphenyl - 3,3′ - (3,3′ - dimethoxy - 4,4′ - biphenylene) ditetrazolium chloride (Nitro-BT), have been used(3,4,5). Introduction of nitro groups into the N-2 phenyl rings of BT (4) conveyed the favorable properties of INT (1) related to its relatively high redox potential(6), and in addition provided a highly substantive, insoluble pigment favorable for demonstrating activity in intracellular organelles(5,7,8). However, on comparing INT and Nitro-BT in both tissue sections and homogenate preparations, certain differences suggested the need for a systematic study of the influence of electronegative groups in the N-3 phenyl ring of 2-*p*-nitrophenyl tetrazolium salts. The 8 monotetrazolium homologues whose preparation is given elsewhere (9), are listed in Table I along with compound IX, which differs from INT (compound III) in having a C-5 methyl group in place of the phenyl group. Nitro-BT (compound X in Table II) was also included.

Materials and methods. Three dehydrogenases (succinic dehydrogenase, SDH; lactic dehydrogenase, LDH; isocitric dehydrogenase, IDH) were tested with the tetrazolium salts (I to X). Rat liver was homogenized (0.1-2 mg/ml) and fresh frozen sections of the same livers were cut in the cryostat at 12 μ. Concentrations of substrates, pH values and duration of incubation were identical in the experiments using both homogenates and sections. Phenazine methosulfate was used as an intermediate electron carrier between the dehydrogenases and the tetrazolium salts whenever homogenates were used. Otherwise, the incubation media used were similar to those described previously(2,5,7,8).

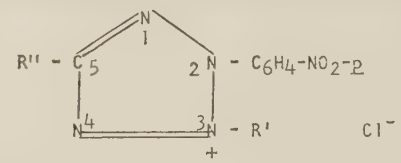
Results. The extent of enzymatic reduction by tissue homogenates of the 9 tetrazolium salts is compared to INT in Table II. Replacement of the iodo group of INT with either a hydrogen atom (I) or an electropositive methoxy group (II) resulted in a marked decrease in rate of reduction by all 3 dehydrogenase systems. A weakly electronegative group, COOH (VI) was only slightly better as an electron acceptor for SDH and IDH, but not for LDH. Although it was expected from electronic theory that chloro (IV) and bromo (V) substitutions would enhance reduction over that of INT, this was not the case. Cyano (VII) and Nitro (VIII) substitutions yielded compounds which were poorer electron acceptors than INT with SDH, equally good acceptors with IDH, and as good as INT (VII) or better than INT (VIII) with LDH. Similarly, Nitro-BT (X) was comparable to INT with LDH and IDH, but only half as good with SDH. The important influence of the C-5 phenyl group was shown by the marked decrease in enzymatic reduction when the phenyl of INT was replaced by a methyl group (IX).

The finding that Nitro-BT accepted electrons at half the rate observed with INT in assay of SDH in liver homogenates was not in agreement with the equally good reduction

* This study was supported by research grants from Nat. Inst. Health, Dept. of H.E.W., Bethesda, Md.

† We acknowledge technical assistance of H. Wasserkrug, J. Goldberg, S. Margulies, M. Davidson, and D. Goldstone.

TABLE I. Tetrazolium Salt Homologues Tested.*



Compound No.	R'	R''
I	C_6H_5	C_6H_5
II	C_6H_4-OCH_3-*p*	"
III	" -I-*p*	"
IV	" -Cl-*p*	"
V	" -Br-*p*	"
VI	" -COOH-*p*	"
VII	" -CN-*p*	"
VIII	" -NO_2-*p*	"
IX	" -I-*p*	CH_3

* INT (III) was obtained commercially. The other tetrazolium salts were prepared in our laboratory(9).

of these 2 reagents in liver sections (Table II). On histochemical comparison of the 10 tetrazoles with the 3 dehydrogenases other discrepancies between the histochemical and biochemical behavior of these reagents was noted. For example, although Nitro-BT (X) is roughly similar to INT (III) with homogenate assays of LDH and IDH, Nitro-BT is a much better electron acceptor than INT in histochemical demonstration of LDH (Table II). A second nitro group (VIII) yields a tetrazole which is more readily reduced histochemically than INT by the 3 dehydrogenases, whereas in the homogenate assays INT is superior to VIII for SDH and comparable for IDH (Table II).

However, recent observations on sites of electron transfer in the succinoxidase system to several tetrazolium salts revealed differences that may help clarify some of these discrepancies(10). The loci of electron transfer in the respiratory chain were identified by use of purified soluble succinic dehydrogenase, inhibition by Antimycin A, removal of cytochrome c, inhibition with cyanide of cytochrome oxidase, and anaerobiosis. Homogenates were selected for study of the disrupted system, and tissue sections provided a means of testing the intact enzyme system. With the aid of phenazine methosulfate, electrons were passed from purified SDH to INT (2,10), in absence of phenazine methosulfate, electrons were passed to INT and Nitro-BT after cytochrome b but before the Antimycin A sensitive factor(10). The tetrazolium salts lacking nitro substituents (BT) received their electrons from reduced cytochrome oxidase (10). Explanations are not readily apparent for all differences in activity between the tetrazolium homologues noted in this study. Although the redox potentials of these tetrazoles are of some importance, it seems unlikely that this can be responsible for all the differences noted. The important issue which these data emphasize is that use of new tetrazolium salts may require prior investigation of sites of electron transfer as compared with those of INT and Nitro-BT, before safe interpretation of results can be made.

Summary. Electronegative groups attached to either the N-2 phenyl or the N-3 phenyl rings are important in determining the readiness of enzymatic transfer of electrons to tetrazolium salts. The magnitude of the effects exerted by nitro, chloro, or cyano moieties are not identical when different dehydrogenase systems are compared. Furthermore, differences noted between several tetrazolium salts in any one dehydrogenase system are not

TABLE II. Biochemical Reduction of Tetrazolium Salts by 3 Dehydrogenase Systems.

Compound No.	SDH[a]	LDH[a]	IDH[a]
	(%)		
I	5	10	11
II	6	10	13
III	100 (2+)	100 (2+)	100 (2+)
IV	47	62	78
V	65	64	50
VI	25	8	29
VII	76	100	98
VIII	40 (3+)	117 (3+)	94 (3+)
IX	23	22	46
X	52 (2+)	92 (4+)	84 (2+)

Compound No. refers to structures given in Table I. Compound X is Nitro-BT. Enzyme source was rat liver homogenate (2 mg/ml), and incubation media were similar to those described previously (2,7,8). Micromoles of formazan (or twice the micromoles of diformazan) produced when the 3 enzyme systems were tested with each tetrazolium salt were converted to per cent using values for INT (III) reduction as 100%. Averages of 3 or more experiments are given. Estimation of color density of histochemical preparations is given in parentheses on a scale of 1+ to 4+. Fresh frozen blocks of rat liver were sectioned at 12 μ and incubated for 15 min. at 37° in the reaction media described previously(5,7,8).

necessarily reproduced when biochemical and histochemical preparations are compared. Although the reasons for these differences are not immediately apparent, preliminary observations with the succinoxidase system suggest that some clarification may follow closer inspection of site of transfer to tetrazolium salts in the chain of electron transport.

1. Atkinson, E. H., Melvin, S., Fox, S. W., *Science,* 1959, v111, 385.

2. Nachlas, M. M., Margulies, S. I., Seligman, A. M., *J. Biol. Chem.,* 1960, v235, 499.

3. Seligman, A. M., Rutenburg, A. M., *Science,* 1951, v113, 317.

4. Tsou, K. C., Cheng, C. S., Nachlas, M. M., Seligman, A. M., *J. Am. Chem. Soc.,* 1956, v78, 6139.

5. Nachlas, M. M., Tsou, K. C., DeSouza, J., Cheng, C. S., Seligman, A. M., *J. Histochem. & Cytochem.,* 1957, v5, 420.

6. Karmarkar, S. S., Pearse, A. G. E., Seligman, A. M., *J. Org. Chem.,* 1960, v25, 575.

7. Nachlas, M. M., Walker, D. G., Seligman, A. M., *J. Biophys. & Biochem. Cytol.,* 1958, v4, 29.

8. ———, *ibid.,* 1958, v4, 467.

9. Karmarkar, S. S., Barrnett, R. J., Nachlas, M. M., Seligman, A. M., *J. Am. Chem. Soc.,* 1959, v81, 3771.

10. Nachlas, M. M., Margulies, S. I., Seligman, A. M., *J. Biol. Chem.,* 1960, in press.

Received April 21, 1960. P.S.E.B.M., 1960, v104.

Role of Local Trauma in Production of Cutaneous Calcinosis by Dihydrotachysterol.* (25855)

H. SELYE, P. JEAN† AND R. VEILLEUX‡

Inst. de Médecine et de Chirurgie expérimentales, Université de Montréal, Montreal, Canada

A cutaneous calcinosis with sclerosis has been produced in rats treated during the first few days of life with parathyroid hormone(1) or with dihydrotachysterol, "DHT"(2), a Vit. D derivative that closely imitates parathyroid hormone in its pharmacologic properties. This skin lesion resembles human scleroderma in many respects. It has also been claimed that, in clinical scleroderma, calcium content of the skin is sometimes considerably increased(3,4) and that partial parathyroidectomy may induce marked improvement(5). Studies designed to clarify the possible interrelations between these clinical and experimental diseases would be greatly facilitated if a suitable laboratory model of this type were readily available. However, the previously described cutaneous lesions produced either with parathyroid hormone or with DHT do not lend themselves well to this kind of study because they: (a) are accompanied by high mortality, (b) develop only on the scalp and neck, (c) occur exclusively during the first few days of life and even then not in all instances. In the internal organs of DHT-treated rats, topical trauma can cause local calcification(6); clinical scleroderma also tends to develop at sites of local injury(7). The object of this communication is to show that, under suitable conditions, cutaneous calcinosis with sclerosis can be produced at will in predetermined regions of the skin, even in adult rats, if the selected area is lightly traumatized at a critical time of systemic DHT treatment.

Methods. For the sake of brevity, we shall not describe the many preliminary experiments necessary to establish optimum conditions for regular production of cutaneous calcinosis by DHT + local trauma. It should be emphasized, however, that DHT is most effective when given in oil, *per os,* and that predisposition for cutaneous calcinosis is greatest after very acute DHT intoxication. In one such experiment, 80 female Sprague-Dawley rats, mean body weight of 100 g (range: 85-115 g), were given 250 μg of DHT

* Supported by Army Medical Research and Development Command, Dept. of U. S. Army, Irwin Strasburger Memorial Medical Fn., and Geigy Pharmaceuticals.

† Fellow of Quebec Heart Fn.

‡ Fellow of National Research Council of Canada.

TABLE I. Role of Local Trauma in Production of Cutaneous Calcinosis by DHT,* in 7 Groups.

Day of epilation	Cutaneous calcinosis (scale: 0-3)	
	Scalp	Back
0	.6 ± .17	0
1	1.0 ± .32	0
2	1.2 ± .20	0
3	2.8 ± .17	.8 ± .54
4	3.0 ±0	2.7 ± .33
5	2.8 ± .17	2.7 ± .33
7	.3 ± .20	.2 ± .17

* All groups were treated with DHT between 2nd and 5th day, as indicated in text. Readings listed here represent the condition on 11th day, when the lesions were maximally developed.

("Calcamin," Wander) in 0.5 ml of corn oil daily on 4 consecutive days. Even without local trauma, 72 of these animals manifested minor spots of cutaneous calcinosis in the scalp, during the first 2 weeks. However, the lesions were barely detectable and never occurred in any other part of the skin. If the total amount of DHT (*i.e.*, 1 mg) is given in a single dose, incidence of the lesions is approximately the same but mortality is high; if the same amount is administered over a longer period (*e.g.*, 100 μg/day during 10 days), incidence of skin lesions drops considerably. These findings furnished the basis for the technic of the experiment to be described here. We wanted to determine: first, whether the minor local trauma of epilation could increase incidence and severity of cutaneous calcinosis in the scalp; second, whether epilation could induce such lesions, even in skin regions where they normally do not result from DHT treatment; and third, whether, in the course of DHT overdosage, there is a "critical period" during which skin trauma is most effective in precipitating cutaneous calcinosis. Forty-two female Sprague-Dawley rats, mean initial body weight of 100 g (range: 90-110 g), were subdivided into 7 equal groups as indicated in Table I. The hair was removed by plucking, from the calvarium and from a circular patch approximately 2 cm in diameter on the back. This was done at different times in the various groups (Table I). DHT was given to all rats by stomach tube, 250 μg in 0.5 ml of corn oil, once daily, from the 2nd to the 5th day. Degree of cutaneous calcinosis was repeatedly appraised in terms of an arbitrary scale: 0 (no lesion), 1 (just detectable lesion), 2 (moderate lesion), and 3 (maximal lesion).

Results. The first macroscopically detectable evidence of cutaneous calcinosis appeared, on the 9th day, in animals whose hair was removed during the last 2 days of DHT treatment. It manifested itself in the form of scattered, indurated, whitish skin plaques, which gradually tended to coalesce. These changes reached a maximum in all groups on the 11th day and the means of the readings made at that time are listed in Table I, together with their standard errors. Evidently, there is a definite "critical period" during the last 2 days of DHT treatment, at which time epilation is most effective in eliciting cutaneous calcinosis.

In rats optimally sensitized by systemic

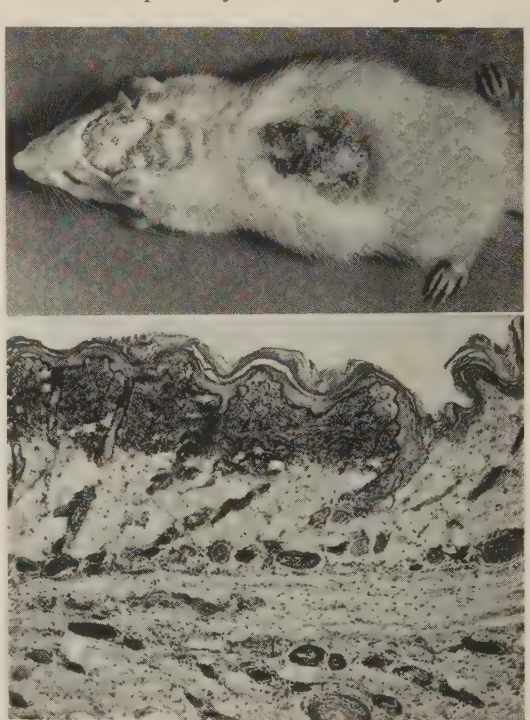


FIG. 1. *Top,* typical cutaneous calcinosis over the epilated area on scalp and back, as it appears when the lesions are maximally developed, in a rat of Group 5 photographed on 11th day. *Bottom,* typical histologic appearance of an early lesion in a rat of Group 5 killed on 8th day. Note that, initially, calcium deposition is limited to the subepithelial connective tissue fibers in a sharply circumscribed region (black granules under left two-thirds of surface). In the deeper layers, there is edema but, as yet, no sclerosis (von Kóssa × 72).

treatment with DHT, epilation greatly aggravates the otherwise inconstant and mild cutaneous calcinosis of the scalp; it can even induce lesions in an area not otherwise affected, such as the back. In both these regions, the skin is transformed into hard, heavily calcified plaques. On histologic sections (fixed in alcohol-formol and stained with von Kóssa's method), calcium deposition is seen mainly in the subepithelial connective tissue fibers and the basement membrane region (Fig. 1). Of course, on von Kóssa stained sections, it is impossible to state with certainty whether the mineral deposits are within or around the structural elements just mentioned. Yet, in following the early stages of tissue calcification, the impression is gained that the collagen fibers swell while they become gradually impregnated with calcium salts. On sections stained with Weigert's elastica stain, it is evident that simultaneously with this calcification in the collagen, the elastic fibers become fragmented. All these changes are reminiscent of human scleroderma, especially the so-called Thibierge-Weissenbach type, which is characterized by calcification.

During the 10 days following their appearance, these plaques are gradually demarcated from the underlying dermis and cast off. Then, cicatrization and sclerosis ensue. After the damaged part is shed, the skin underneath becomes re-epithelialized but remains partly or completely hairless during the following 3 weeks of observation.

Summary. In rats, a cutaneous calcinosis with sclerosis, not unlike that seen in certain types of clinical scleroderma, can be produced at will in predetermined regions of the skin. This is best accomplished if, at a critical time of systemic dihydrotachysterol overdosage, the selected cutaneous area is lightly traumatized by epilation.

1. Selye, H., *J.A.M.A.*, 1932, v99, 108.
2. ———, *J. Invest. Dermat.*, 1957, v29, 9.
3. Leriche, R., Jung, A., De Bakey, M. E., *Surgery*, 1937, v1, 6.
4. Leriche, R., Jung, A., Sureyya, C., *Presse méd.*, 1935, v43, 777.
5. Leriche, R., Jung, A., *ibid.*, 1935, v43, 1361
6. Selye, H., *Z. Urol.*, 1957, v50, 440.
7. Selye, H., *Virchows Arch. f. path. Anat.*, 1932, v286, 91.

Received April 25, 1960. P.S.E.B.M., 1960, v104.

Biological Activity of Blood Insulin Complexes Examined by Rat Diaphragm Tissue Assay.* (25856)

KARE GUNDERSEN† AND HARRY N. ANTONIADES (Introduced by D. M. Surgenor)
Boston Dispensary and Dept. of Preventive Medicine, Tufts School of Medicine, Boston, and Protein Fn. Labs., Jamaica Plain, Mass.

Antoniades *et al.*(1-3) suggested that insulin in blood and pancreas is largely bound by other proteins and, more specifically, by basic proteins. The insulin-basic protein complexes could be adsorbed on cationic exchange resin, in the sodium form, and could be extracted from the resin by elution with acid or alkali(3). The present communication describes studies on biological activity of insulin-complexes extracted from human blood with use of cationic exchange resin (Na^+ form). Two types of preparations have been examined for insulin-like activity by the rat diaphragm tissue assay as described by Vallance-Owen and Hurlock(4). The data indicated that the biological effect of insulin in its complex form examined *in vitro* by the diaphragm assay differs significantly from the biological effect of insulin in the "free" form, liberated from the complex at pH 9.8. Preliminary data suggest that, in contrast to the rat diaphragm assay, adipose tissue assay can

* This work was supported by grants from National Foundation and Eli Lilly and Co.

† Recipient of research fellowship, Nat. Inst. of Arthritis and Metab. Dis.

TABLE I. Bioassay of Resin Eluates by Rat Diaphragm Tissue Assay.*

	Before pH change		After pH change	
Lot No.	Glucose uptake in μg/10 mg dry diaphragm $\pm$ SE	Insulin-like activity in mu/l of plasma	Glucose uptake in μg/10 mg dry diaphragm $\pm$ SE	Insulin-like activity in mu/l of plasma
016	42 $\pm$ 18	1.35	184 $\pm$ 25	32.9
	–82 $\pm$ 10	.0	204 $\pm$ 29	82.6
	–96 $\pm$ 5	.0		
	58 $\pm$ 29	.486		
	2† $\pm$ 4	.0	116 $\pm$ 8	14.0§
	4† $\pm$ 5	.0	106 $\pm$ 7	20.2§
	10 $\pm$ 10	.0		
			264 $\pm$ 11	83.4
			152 $\pm$ 12	35.1
		(48.6)‡		
		(40.5)‡		
052	6 $\pm$ 11	.0	278 $\pm$ 13	51
		(20)‡		

* All determinations were made in triplicate and calculated as net glucose uptake above control (buffer alone).

† Dialyzed against Gey and Gey's bicarbonate buffer(6) overnight.

‡ Activity in parentheses obtained on same sample by rat adipose tissue assay. Assays for insulin-like activity were kindly carried out by Drs. A. E. Renold and J. Steinke.

§ Low recoveries may result from incomplete dissociation of the complex or high pH values. pH adjustment was made without use of continuous recording pH meter.

determine insulin in its complex form.

Materials and methods. Resin eluate, lot 016 has been prepared by acid elution (0.1 N H_2SO_4, pH 2.0) from cationic exchange resin (Na^+ form) following blood collection(2). Resin eluate, lot 052 has been prepared by elution with 0.1 N NH_4OH, pH 10.5(3). The pH of the eluates was adjusted to 7.2 followed by dialysis against 0.02 M NaCl and lyophilization. *Rat diaphragm tissue assay*: based on increment in glucose uptake by this tissue in presence of insulin(4). *Rat adipose tissue assay*: based on effect of insulin in production of $C^{14}O_2$ from glucose-C^{14} by this tissue(5). *Dissociation of insulin from its complex* in eluate concentrates was carried out at pH 9.8. Lyophilized resin eluates containing the insulin complexes were dissolved in Krebs' bicarbonate buffer or in Gey and Gey's bicarbonate buffer(6). pH of the solution was brought to 9.8 under continuous stirring with use of 0.5 N NaOH, which was added slowly and dropwise. A continuous recording pH meter (Radiometer, Copenhagen, Denmark) was employed in these studies, and particular care was exercised not to exceed pH 10. The mixture was centrifuged at 4°C for 30 minutes at 3000 rpm, and pH of the supernatant fluid was adjusted to 7.2. The supernatant fluid was dialyzed overnight against the buffer employed in the assay and was examined for insulin-like activity. Control experiments of the untreated eluate, dialyzed against the same buffer were carried out simultaneously.

Results. Table I shows the results. The insulin-like activity in its complex form does not exhibit full biological activity on the rat diaphragm tissue assay. Insulin-like activity was demonstrated upon liberation of insulin from its complex by centrifuging at pH 9.8.

Biological assays for insulin-like activity of identical untreated preparations carried out by the rat adipose tissue assay(5) showed that this assay could determine insulin-like activity in its complex form (Table I). Although the reasons for the differences between the 2 assays are still obscure, one may speculate that adipose tissue can utilize insulin in its complex form whereas such complexes are not utilized by rat diaphragm tissue.

These observations have been made in an isolated and highly purified system. It is possible that in blood serum, as in the resin eluates, different fractions of insulin-like activity are determined by the different assays employed for estimation of the insulin-like activity.

Summary. Insulin complexes prepared from human blood showed no significant in-

sulin activity when such preparations were examined by the rat diaphragm tissue assay. After dissociation of insulin from its complex(es) at pH 9.8 insulin-like activity could be demonstrated by this assay. Identical preparations examined by the rat adipose tissue assay showed that this tissue could manifest insulin-like activity in the presence of the insulin complex itself. A difference is thus evident in the mechanism of utilization of the insulin complex(es) by these 2 different tissues.

1. Antoniades, H. N., Beigelman, P. M., Pennell, R. B., Thorn, G. W., Oncley, J. L., *Metabolism: Clin. and Exp.,* 1958, v7, 266.

2. Antoniades, H. N., *Science,* 1958, v127, 593.

3. Antoniades, H. N., Renold, A. E., Dagenais, Y. M., Steinke, J., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 677.

4. Vallance-Owen, J., Hurlock, B., *Lancet,* 1954, v1, 68.

5. Martin, D. B., Dagenais, Y. M., Renold, A. E., *Lancet,* 1958, v2, 76.

6. Gey, G. O., Gey, M. K., *Amer. J. of Cancer,* 1936, v27, 45.

Received April 27, 1960. P.S.E.B.M., 1960, v104.

Transplantability Changes of Mouse Mammary Tumors After Passage Through Tolerant Homologous Recipients.* (25857)

CARLOS MARTINEZ, HARVEY KELMAN AND ROBERT A. GOOD
Depts. of Physiology and Pediatrics, University of Minnesota Medical School, Minneapolis

Transplantation of normal and neoplastic tissues in highly inbred strains of mice is ruled by classical principles of inheritance. Tumors taken from animals of one inbred strain can be successfully transplanted into the same individual (autologous graft) or into members of the same strain (isologous grafts). In addition, by crossing mice of 2 different strains, the resulting F_1 hybrids accept tumor transplants taken from individuals of either parent strain. Finally, F_2 hybrids or backcross hybrids to the non-susceptible parent accept the tumor with a frequency which will depend on number of histocompatibility factors required by the particular tumor employed (See review by Little)(1). Barrett and Deringer first reported that after passing a tumor through F_1 hybrids produced by outcrossing the strain of origin times another resistant strain, transplantability of this tumor usually increased when tested in F_2 or backcross hybrids between the same 2 strains used to produce the intermediate F_1 cross(2). The change was demonstrated to be permanent even when the tumor was returned to the original strain(3) and quite specific for the particular genotype employed in the F_1 passage (4). This phenomenon first explained by its discoverers as due to "adaptation" of the tumor, was later thought to be due to "immunoselection" of certain simplified antigenic types of cell populations preexisting in the tumor(5). However, E. and G. Klein(6,7) could not confirm cell selection interpretation. Experiments using preimmunized animals led these investigators to favor the hypothesis that the change in transplantability induced by passage through the F_1 hybrid might be the result of a change in response of the tumor to specific isoantibodies it provokes in the F_1 individual. They also found that the Barrett and Deringer phenomenon could be induced in tumor cells enclosed in cell impermeable diffusion chambers placed into the peritoneal cavity of F_1 hybrid individuals. From these experiments they concluded that the F_1 hybrid effect is probably humoral in nature(8). Using the phenomenon of acquired immunological tolerance we have studied transplantability changes of tumors after passing these tumors through homologous mice previously made tolerant of the strain of animals from which the tumors derived, and compared these results with those

* Aided by grants from Minn. Division, Am. Cancer Soc. and USPHS.

obtained after passage through the corresponding F_1 hybrid. Studies were also conducted to ascertain whether or not similar transplantability changes would be obtained if the intermediate host was either an homologous neonate or an isologous individual made previously tolerant to F_1 hybrid strain tissue. Results of these experiments are herein reported.

Method. Two experiments were performed. In one the tumor used was a mammary adenocarcinoma appearing spontaneously in a breeder female mouse of the A strain. This tumor was first transplanted into small groups of mice of the A, AZ F_1 hybrids, Z mice previously made tolerant of AZ F_1 hybrid tissue by injection with AZ F_1 cells in the neonatal period and A animals previously made tolerant of AZ F_1 hybrid tissue. Approximately 40 days after first tumor inoculation a newly developed tumor was removed from one mouse of each preceding group and retransplanted into $(AZF_1 \times AZF_1)$ F_2, $(AZF_1 \times A)$ BC and $(AZF_1 \times Z)$ BC. In these groups, incidence of tumor takes ending in death of the host was determined. Tolerant animals were prepared by the method recommended by Billingham & Brent(9) consisting of intravenous injection of prospective recipient animals with viable spleen cells taken from individuals of the prospective donor strain during neonatal period (0-24 hrs after delivery). Before tumor inoculation the existence of a state of tolerance was established by demonstrating survival of a skin homograft taken from a homologous donor of the same strain which had contributed the spleen cells. In all instances tumors were transplanted subcutaneously into the right groin by injecting .2 cc of a 10% tumor cell suspension prepared by cutting the tumor into small pieces with scissors and grinding these pieces in a glass homogenizer. In the second experiment the tumor used was a mammary adenocarcinoma which originally appeared in an A breeder mouse. The tumor was subsequently maintained in the laboratory for 3 successive transfers carried out in mice of the strain of origin. The intermediate hosts were: A, $(A \times Z)F_1$ hybrids, A mice previously made tolerant of $(A \times Z)F_1$ hybrid tissue and Z newborn mice. The latter group received the tumor transplant as neonates (0-24 hr after birth) by introducing a bit of tumor tissue subcutaneously through a small incision in the skin of the back, followed by closure of the wound with a single silk suture. When this procedure was used most of the animals grew the transplanted homologous tumor(10). Here again approximately 40 days after inoculation a newly developed tumor was removed from one mouse of each group and retransplanted by subcutaneous inoculation of a 10% tumor cell suspension into groups of $(AZF_1 \times AZF_1)$ F_2 hybrids. As in the first experiment, incidence of tumor takes ending in death of the host was determined in each group of F_2 hybrids.

Results. Results are recorded in Tables I and II. In the first experiment (Table I) when intermediate hosts were mice of the same strain as that contributing the tumor, incidence of tumor takes in AZ F_2 hybrids was only 2% (1/44); in back-cross hybrids to the susceptible parent (ABC) incidence of tumor takes was 90% (20/22) and when the secondary hosts were back-cross hybrids to the non-susceptible parent (ZBC) no takes were observed (0/44). When intermediate hosts had been AZ F_1 hybrids, incidence of takes in AZ F_2 increased to 23% (11/48); all ABC hybrids accepted this tumor and 10% (4/40) of the ZBC hybrids were susceptible.

When intermediate hosts had been homologous Z strain mice previously made tolerant

TABLE I. Barrett-Deringer Effect on Spontaneous Mammary Adenocarcinoma.

Original tumor	1st host	2nd host	# of takes in 2nd host*	%
A Spont. #1	A	AZF_2	1/44	2
Idem	"	ABC	20/22	90
"	"	ZBC	0/44	0
"	AZF_1	AZF_2	11/48	23
"	"	ABC	23/23	100
"	"	ZBC	4/40	10
"	Z tol. AZF_1	AZF_2	15/55	27
"	"	ABC	30/32	94
"	"	ZBC	2/42	5
"	A tol. AZF_1	AZF_2	7/49	14
"	"	ABC	19/19	100
"	"	ZBC	2/19	11

* No. +/No. of grafted.

TABLE II. Barrett-Deringer Effect in F_1 Tolerant and Newborn Mice.

Original tumor	1st host	2nd host	# of takes in 2nd host*	%
A (3rd gen.) #2	A	AZ F_2	1/20	5
Idem	AZ F_1	"	6/27	22
"	A tol. AZ F_1	"	8/30	27
"	Z newborn	"	14/27	52

* No. +/No. of grafted.

of AZ F_1 hybrid tissue, incidence of tumor takes was 27% (15/55) in AZ F_2 hybrids, 94% in ABC hybrids and 5% in ZBC hybrids. Finally, when intermediate hosts were A strain isologous mice made tolerant of AZ F_1 tissue, incidence of tumor takes in the AZ F_2 was 14% (7/49); in ABC mice, 100% (19/19), and in ZBC mice, 11% (2/19).

In the second experiment in which the tumor was a mammary adenocarcinoma which had been passed 3 times (Table II) the results were as follows: When intermediate hosts had been of same strain as the tumor, incidence of takes obtained in AZ F_2 hybrids was 5% (1/20). Incidence of tumor takes increased to 22% (6/27), when intermediate recipients had been AZ F_1 hybrids, to 27% (8/30) when the tumor had first been transferred to isologous A mice tolerant of AZ F_1 tissues and, finally increased to 52% (14/27) when intermediate hosts were homologous Z strain mice which tolerated the tumor by virtue of the fact that transplantation was accomplished at birth.

Discussion. These results are of interest from several points of view. First, they confirm the observations of Barrett and Deringer(2), who discovered that changes occurred in transplantability of repeatedly transplanted tumors following passage through F_1 hybrid mice produced by outcrossing of the tumor bearing strain with another resistant strain. In our experiments a similar effect of passage through F_1 hybrids was observed in spontaneous mammary adenocarcinoma which had not previously been transplanted and in tumors which had previously been subjected to only 3 successive transfers within strain of origin. In the first experiment, transfer of an A strain tumor first to isologous A mice and later into AZ F_2 hybrids gave a 2% incidence of takes in the latter animals indicating the expression of approximately 13 histocompatibility factors ($0.75^{13} = 0.02$). However, when the tumor was first transplanted to AZ F_1 hybrids, incidence of takes obtained in AZ F_2 hybrids was 23% indicating that number of histocompatibility factors being expressed had dropped considerably from approximately 13 to approximately 5 ($0.76^5 = 0.23$).

Similar results were obtained in the second experiment although in this instance the histocompatibility factors operating decreased from approximately 10 ($0.75^{10} = 0.06$) to 5 ($0.75^5 = 0.23$) after F_1 hybrid passage.

Of particular interest are the findings regarding previous passage of the tumor through animals of the Z homologous strain rendered tolerant of the tumor either by injection of AZ F_1 spleen cells (first experiment) or by transplanting the tumor during neonatal period (second experiment).

In both instances incidence of takes of these tumors when tested in AZ F_2 hybrids increased as compared to that observed after previous residence in isologous hosts, indicating that the foreign environment to which either tumor was subjected induced changes in transplantability which are the same as those induced after the tumor had been grown in the F_1 environment.

Klein has pointed out(8) that the F_1 hybrid effect as well as the effect on tumors by passing the tumor through homologous individuals in which the homograft reaction is not effective, *i.e.*, by direct transfer of the tumor to embryos of a homologous strain(11) or by transfer to previously irradiated animals (12) may have analogous mechanisms. Our results indicate that homologous mice made immunologically tolerant of the tumor by spleen injection at birth also provide an environment which acts upon tumors transplanted to them to induce the Barrett and Deringer phenomenon.

In the second experiment the magnitude of the change in transplantability of the tumor when the latter had been implanted in newborn homologous Z strain mice was greater than that obtained after AZ F_1 passage. This

might possibly be fortuitous or may be due to the genetic difference between these 2 types of recipients. It is conceivable that the Z newborn individual which tolerates the homologous tumor by virtue of its immunological nullness provides an environment more conducive to adaptation of the tumor because the foreign Z environment differs more markedly from the A tumor than does the AZ F_1 hybrid environment.

Changes in transplantability of the A strain tumor were also induced by passage through A strain mice previously made tolerant of homologous AZ F_1 hybrid tissue. These results are even more difficult of interpretation. However, if one assumes that immunological tolerance induced by intravenous injection of homologous spleen cells or by other methods depends upon constant presence of foreign antigens in residence and circulating in the blood of the tolerant host, perhaps in the form of intact cells(13), then it follows that under the conditions of these experiments growth of the tumor takes place in an individual having circulating reticulo-endothelial cells of its own as well as those of the AZ F_1 hybrid. Therefore, in the tolerant isologous animal the tumor is being constantly subjected to a humoral or cellular environment which might be quite similar to that offered to the tumor by the F_1 hybrid itself.

Summary. 1. Modification of transplantability of tumors by passage through F_1 hybrid as originally reported by Barrett and Deringer has been confirmed. 2. Modification of transplantability of tumors induced by foreign environment holds for spontaneous mammary adenocarcinoma and for mammary adenocarcinoma, passaged a few times. 3. A similar modification of transplantability occurs when A strain tumor has been passed through homologous host made tolerant of A strain tissue by intravenous injection of spleen cells at birth. 4. Modification of transplantability of tumors is also induced by passage of tumor through isologous hosts made tolerant of F_1 hybrid homologous strain by injection of homologous spleen cells in neonatal period. 5. The most striking modification of transplantability of mammary adenocarcinoma was obtained by passage through homologous (foreign) hosts at birth. The physiological and genetic basis for alteration of transplantable tumors in these and previous studies remains obscure.

1. Little, C. C., In *Biology of the Laboratory Mouse,* Chap. 7, Blakiston Co., Philadelphia, 1941.

2. Barrett, M. K., Deringer, M. K., *J. Nat. Cancer Inst.,* 1950, v11, 51.

3. ———, *ibid.,* 1952, v12, 1011.

4. Barrett, M. K., Deringer, M. K., Hansen, W. H., *ibid.,* 1953, v14, 381.

5. Hauschka, T. S., *ibid.,* 1953, v14, 723.

6. Klein, E., Klein, G., *Transpl. Bull.,* 1956, v3, 136.

7. Klein, G., Klein, E., *Symposia Soc. Exp. Biol.,* 1957, v11, 305.

8. Klein, G., *Canadian Cancer Conf.,* 1959, v3, 215.

9. Billingham, R. E., Brent, L., *Transpl. Bull.,* 1957, v4, 67.

10. Aust, J. B., Martinez, C., Bittner, J. J., Good, R. A., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 27.

11. Koprowski, H., Theis, G., Love, R., *Proc. Roy. Soc.,* 1956, vB 146, 37.

12. Krebs, C., Thodarson, O., Harbo, J., *Acta Radiol.,* 1942 Suppl. v44.

13. Martinez, C., Smith, J. M., Aust, J. B., Mariani, T., Good, R. A., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 640.

Received April 27, 1960. P.S.E.B.M., 1960, v104.

Response of Rat Zona Glomerulosa in Experimental Nephrosis.* (25858)

HELEN WENDLER DEANE, ROBERT H. SCHNEIWEISS AND LEWIS I. GIDEZ†
(Introduced by Howard A. Eder)
Depts. of Pathology, Anatomy, Biochemistry, Medicine and Radiology, Albert Einstein College of Medicine, N. Y.

Hypertrophy and activation of the adrenal zona glomerulosa occur in experimental situations designed to increase output of electrolyte-regulating corticosteroid; Na^+ deprivation was an excellent stimulus(1). Subsequently aldosterone was found to be the most active mineralocorticoid(2); its synthesis occurs almost exclusively in the glomerulosa (3). Secretion of aldosterone is enhanced by reduction of Na^+ intake and also in diseases characterized by edema and ascites(4), including experimental nephrosis in the rat(5,6). Such diseases generally exhibit a demonstrable reduction in plasma Na^+/K^+ ratio(7,8). The present experiment was performed to follow response of glomerulosa during development of and recovery from the nephrotic syndrome induced in rats by aminonucleoside of Puromycin, 6 - dimethylamino - 9 - (3′ - amino - 3′-deoxy-β-D-ribofuranosyl) purine(9).

Methods. Twenty-one young male rats (100 g, Holtzman strain) were given 13 daily subcutaneous injections of 0.5% solution of aminonucleoside‡ in physiological saline, 0.03 ml/10 g body weight(9). Four controls received equivalent injections of saline. Experimental rats were killed at 13, 15, 19, 26, 36 and 61 days after beginning of treatment; controls were killed at 13, 19, 26 and 36 days. The animals were weighed, blood drawn for plasma cholesterol determination(10), and pieces of kidney fixed for microscopic examination. The 2 adrenal glands were trimmed, weighed, and fixed (a) in alcohol-formalin-acetic acid, sectioned serially at 10 μ in paraffin and stained with hematoxylin and eosin, and (b) in formalin, sectioned at 15 μ on freezing microtome and stained with Oil red O.

Results. Weight gain in experimental rats became slower than normal on day 6. They developed gross edema by day 11 and visible ascites by day 13. Maximal accumulation of ascitic fluid occurred between days 20 and 25. From one animal sacrificed on day 19, 21 ml fluid was easily withdrawn from abdominal cavity, and there was additional fluid in thoracic and pericardial cavities. Nine animals died between 15 and 22 days. The remaining ones had diuresis. Lowest body weights were recorded between days 22 and 28, after which the animals regained weight.

Kidney lesions (swelling and pallor, cast formation and internal hydronephrosis, disruption of brush borders, appearance of free lipide droplets in proximal tubular cells) were maximal in rats killed at 19 days and had essentially disappeared by 2 months. At this time, however, some glomeruli appeared contracted and cells therein exhibited fatty infiltration. Plasma cholesterol levels were above normal in all experimental animals, reaching a peak in 15-26 days and remaining above normal after 2 months (Table I).

Width of zona glomerulosa in each animal was determined by making 4 measurements with an ocular micrometer at 560 X on each of 2 sections (paraffin) in mid-region of the gland. At 15 days average width was over 50% above that in controls; thereafter it slowly declined to normal (Table I). Even when most enlarged, the glomerulosa retained abundant lipide (Fig. 1).

Discussion. If administered for longer

* Report represents a portion of a student experiment in Pathology. We express appreciation to other members of the group who participated in experimentation: N. M. Barton, W. D. Gershwin, A. F. Goldberg, G. H. Gottesman, D. A. Inkles, J. Klerer, B. Masek, W. A. Peters and C. P. Weber. Histological preparations were made by Ellen C. Driks, cholesterol determinations by Ann Lorenz.

† This work was done during tenure of Investigatorship of Am. Heart Assn.

‡ Aminonucleoside was made available through generosity of Dr. R. B. Angier of Lederle Labs, American Cyanamid Co., Pearl River, N. Y.

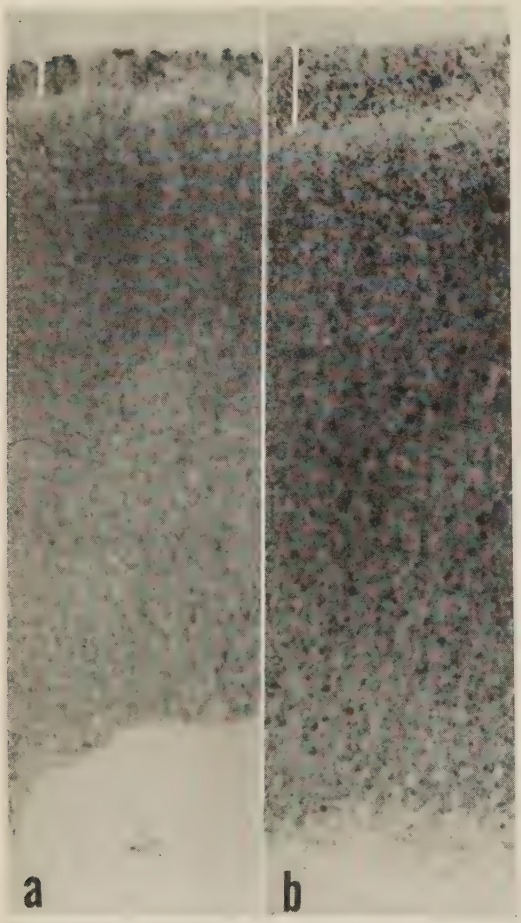


FIG. 1. Frozen sections of adrenal glands, stained with Oil red O, × 100. Width of glomerulosa is indicated on each micrograph. (a) From control killed on day 19; (b) from experimental killed on day 15. In (b) the glomerulosa is significantly broader than normal but its cells contain abundant lipide droplets.

than 12-13 days, the dose of aminonucleoside used is invariably fatal. In this experiment, about half the treated rats died in the severe stage of nephrotic syndrome.

Greatest hypertrophy of glomerulosa was recorded at 15 days after initiation of treatment with aminonucleoside, several days prior to maximal accumulation of ascitic fluid in the surviving group. This result agrees with current evidence that increased secretion of aldosterone may be largely responsible for retention of fluid in diseases like nephrosis (6). Administration of a spirolactone antagonist of aldosterone action on the kidney induces diuresis in nephrotic rats with ascites (11), although it has little or no effect on other important aspects of the disease, such as renal protein loss, shift in plasma albumin/globulin ratio or increased plasma cholesterol. Our results also suggest that regression of the glomerulosa and hence reduction of aldosterone secretion precedes spontaneous diuresis (12). The stimulus to enhanced aldosterone secretion may well lie in the depressed Na^+/K^+ ratio in the plasma (8), although the origin of this depression remains unclear.

Increase in glomerulosal width cannot represent passive response to reduction in width of the entire cortex, as it does after hypophysectomy (1). Indeed, the glands were slightly enlarged, relative to body weight, in experimental rats. Because of fluid retention, this enlargement would have been even more conspicuous if dry body weight had been used for determination of relative size of the glands. Hypertrophy of the glands correlates well with the increased secretion of corticosterone demonstrated by Das Gupta and Giroud (6, 11) and is in contrast with the conclusion of Fiegelson *et al.* (13).

Hypertrophy of the glomerulosa was not so great as found in other, more chronic experimental situations (1). Nor did the zone become depleted of lipide. It may therefore be concluded that the stimulus to secretion was less than maximal.

Recorded for the first time is the gradual morphological repair of the kidney in animals recovered from experimental nephrosis. In this experiment, possibly either because animals were treated for 13 rather than 12 days or because they were older and heavier when the experiment began, plasma cholesterol levels rose higher and dropped more slowly than in an earlier study (14).

Summary. In rats rendered nephrotic by repeated injections of the aminonucleoside of Puromycin, the adrenocortical glomerulosa became hypertrophied. Greatest enlargement occurred on 15th day of experiment, prior to maximal accumulation of ascitic fluid in other animals in the group. Diuresis in surviving animals occurred after glomerulosa had begun to shrink.

TABLE I. Plasma Cholesterol and Adrenal Data for Control and Experimental Rats.

No. animals	Day	Wt, g	Plasma cholesterol, mg/100 ml	Wt adrenals mg	Wt adrenals mg/100 g	Glomerulosal width, ocular micrometer units
			Control animals			
1	13	181	75	37	20	18
1	19	180	64	28	16	18
1	26	214	58	39	18	18
1	36	365	61	54	15	18
			Experimental animals			
1	13	147*	324	31	21	26
2	15	154*	452	34	22	30
3	19	175*	401	39	22	25
2	26	162†	492	41	25	22
2	36	193	161	44	23	18
2	61	301	98			20

* These animals exhibited ascites and tissue edema, so that body weights are too high and relative adrenal weights too low.

† One of these animals had a diuresis on day 25 and still exhibited considerable tissue edema.

1. Deane, H. W., in *Pituitary-Adrenal Function,* R. C. Christman, Ed., A.A.A.S., Washington, D.C., 1951, p31.

2. Simpson, S. A., Tait, J. F., *Recent Progr. Hormone Res.,* 1955, v11, 183.

3. Giroud, C. J. P., Stachenko, J., Piletta, P., in *An International Symposium on Aldosterone,* A. F. Muller and C. M. O'Connor, Eds., Little, Brown, Boston, 1958, p56.

4. Farrell, G., *Physiol. Rev.,* 1958, v38, 709.

5. Singer, B., *Endocrinology,* 1957, v60, 420.

6. Das Gupta, D., Giroud, C. J. P., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 334.

7. Welt, L. G., *Clinical Disorders of Hydration and Acid-Base Equilibrium,* Little, Brown, Boston, 1955, chap. 14.

8. Wolff, H. P., Koczorek, K. R., Buchborn, E., in *An International Symposium on Aldosterone,* A. F. Muller, and C. M. O'Connor, Eds., Little, Brown, Boston, 1958, p.193.

9. Frenk, S., Antonowicz, I., Craig, J. M., Metcoff, J., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 424.

10. Abell, L. L., Levy, B. B., Brodie, B. B., Kendall, F. E., *J. Biol. Chem.,* 1952, v195, 357.

11. Das Gupta, D., Giroud, C. J. P., *Endocrinology,* 1959, v65, 500.

12. Laragh, J. H., *J. Chron. Dis.,* 1960, v11, 292.

13. Fiegelson, E. B., Drake, J. W., Recant, L., *J. Lab. Clin. Med.,* 1957, v50, 437.

14. Gidez, L. I., James, J. A., in *3rd International Conf. on Biochemical Problems of Lipids,* 1956, p354.

Received April 28, 1960. P.S.E.B.M., 1960, v104.

Survival of Polioviruses at Elevated Temperatures (60°-75°C).* (25859)

DONALD N. MEDEARIS, JR.,† JOHN H. ARNOLD‡ AND JOHN F. ENDERS

Research Division of Infectious Diseases, Children's Hospital Medical Center, and Dept. of Bacteriology and Immunology, Harvard Medical School, Boston.

It is generally stated that poliovirus is rapidly destroyed at temperatures slightly exceeding 60°C(1,2). Kaplan and Melnick (3) reviewed the relevant literature and added results of their own showing that even when suspended in milk or cream, which exhibit a stabilizing effect, poliovirus from human feces as well as stock strains of rodent-adapted viruses were inactivated after heating for 30 minutes at 61.7°C. Infectivity of such preparations was also eliminated by heating at 71.1°C for much shorter intervals

* Supported by Research Grant from Nat. Inst. of Allergy and Infect. Dis., N.I.H., U.S.P.H.S.

† Present address: Dept. of Pediatrics, Johns Hopkins Hosp., Baltimore, Md.

‡ Present address: Univ. of North Carolina School of Med., Chapel Hill, N. C.

(15-18 sec). Subsequently Stanley and his co-workers(4,5), Youngner(6) and Lwoff(7) reported that at lower temperatures variant forms exist in stock suspensions of poliovirus which differ markedly in respect to thermosensitivity. In this communication results will be presented that reveal the presence of variants more thermoresistant than those previously described among populations of all 3 types of poliovirus. These results were obtained in a series of experiments initiated after unexpected multiplication of poliovirus Type II had been encountered in safety testing of a suspension heated at 65°C. It had been intended to employ this suspension as antigen in skin tests on human beings.

Procedures. Cell cultures. Primary tube and bottle cultures(8) of trypsinized human amnion and monkey kidney cells§ were incubated at 35°-37°C. Tube cultures were maintained in a roller wheel. Cultures of amnion cells when inoculated were more than 14 days old(9). For cell outgrowth either bovine amniotic fluid medium (BAF)(10) or Melnick's lactalbumin hydrolysate(1) medium were used. For maintenance, only BAF was employed. *Stock suspensions of virus.* Bottle cultures of monkey kidney cell cultures were seeded with undiluted suspensions of Mahoney (Type I), MEF_1 (Type II) and Saukett (Type III) viruses, and 3 serial passages of each were carried out. Prior to inoculation media used for outgrowth were withdrawn and replaced with medium 199. In the 2nd and 3rd passages at time of this substitution the cells were washed once and 5 times respectively with Hanks' solution or medium 199. Cultures employed in preparation of viruses for Exp. 3-6 were, in addition, washed once after inoculation and adsorption of virus. Stock suspensions consisted of fluids of the 3rd passage that were pooled and centrifuged at 2,000 rpm for 30 minutes at 4°C. Aliquots of 0.5-1.0 ml were distributed in ampoules which were glass-sealed and stored in the CO_2 box. As judged by color of the medium, pH of these aliquots did not vary beyond the range of 7-7.6. *Heating.* One of 2 water baths was employed, depending upon temperature range desired. In experiments in which effect of temperatures of 65°C or lower was studied an Elconap model 420-1‖ of large water capacity was used. Higher temperatures were provided by an Electric Hotpack bath model No. 301¶ with small water capacity. Temperature of bath was checked at 3 different loci. Readings of the 3 thermometers did not vary by more than 0.5°C. One of them was calibrated and found to be accurate within 0.05°C. Bulb and adjacent 5 cm of this thermometer were placed in a vessel comparable to that containing the virus suspension. This vessel was sealed at the point where the thermometer extended beyond the lip. Heating of viral suspensions was effected as follows. A number of ampoules were rapidly thawed in the hand and placed in an ice bath along with the calibrated thermometer. These materials were then completely immersed in the water bath at desired temperature. Time and temperature of exposure as indicated by the calibrated thermometer and checked by the 2 others were recorded. Ampoules were then replaced in the ice bath until contents were added to cell cultures. *Inoculation and maintenance of test cultures.* For each determination, contents of 2 ampoules were pooled and aliquots tested for presence of virus. As routine, amnion cell cultures were each inoculated with 0.1 ml of undiluted fluid. In one experiment 0.6 to 1.5 ml was tested. Cultures were observed at intervals for at least 53 days. Nutrient fluid (BAF) was replaced whenever pH fell below 7.0. Fluids from cultures showing questionable cytopathic changes were tested for infective virus by addition to fresh amnion cultures. In one experiment (No. 2) fluids from cultures exhibiting no cellular changes were also tested with negative results. *Controls.* In each experiment uninoculated control cultures ranging in number from 3 to 12 were included. These were treated in the same manner as inoculated cultures. In none was evidence of viral multiplication observed. *Viral assay.* Serial 10-fold

§ Cell suspensions obtained from Okatie Farms, Bluffton, S. C.

‖ Electric Heat Control Apparatus, Newark, N. J.

¶ Electric Hotpack Co.

TABLE I. Thermal Inactivation Poliovirus Type II.

	Temperature													
	55°C				60°C				65°C				75°C	85°C
	Time exposed (min.)													
Exp. No.	10	20	30	60	10	20	30	60	10	20	30	60	60	60
1*	7/12‡	4/10	4/10	1/10	1/10	0/10	0/10	0/10	1/10	0/10	1/10	4/10§		
2*	15/15			12/15	1/15			1/15	3/15			1/15		
3†												1/15		
4†													1/15§	
5†														0/15
6†													0/3 ‖	

* Titer of virus prior to heating: $10^{7.3}$ TCD_{50}/ml.
† *Idem* $10^{6.5}$ ".
‡ No. tubes positive/No. tubes inoculated.
§ Identified by neutralization test.
‖ Each tube inoculated with 0.6-1.5 ml rather than with 0.1 ml of heated suspension.

dilutions of the suspension were prepared in Hanks' solution or medium 199. Each of 5 replicate tube cultures of amnion cells was inoculated with 0.1 ml of each dilution. Infectivity titers were expressed as the log TCD_{50}/ml of the undiluted suspension. Plaque morphology was studied in bottle cultures of amnion cells following procedure described by Frothingham(8). *Neutralization tests* were done in amnion cultures employing 100 TCD_{50} of virus and a final volume of diluted type specific poliovirus antiserum and virus suspension of 0.2 ml.

Results. The results of heating 2 different suspensions of Poliovirus Type II at different temperatures for varying intervals are summarized in Table I. Evidently viral infectivity was not completely destroyed within the temperature range 55-75°C when heated for 1 hour—the longest interval studied.

Comparable experiments were carried out with suspensions of Type I and III polioviruses but temperature range was limited to 65°-85°C. Again it became apparent (Table II) that very small amounts of virus may remain capable of initiating infection of cell cultures after heating at 65°C for 1 hour. No evidence of survival, however, was obtained after heating aliquots of same suspensions for 1 hour at 75° or 85°C.

That exposure to temperatures of 55°-65°C was not without effect on surviving viral units was indicated by prolongation of the interval between inoculation and first appearance of cytopathic changes in infected cells. Occasionally CPE was first noted only after the third to the fifth week (Table III). In virus exposed to 55°C about one-third of the cultures exhibited delayed CPE. Contrasting sharply with these findings was prompt appearance of CPE in cultures inoculated with unheated virus. Thus in repeated titrations of the stock suspensions CPE always became evident at the endpoint dilutions within 6 days.

That the greatly enhanced resistance of these rare variants to heat might be genetically determined is an obvious hypothesis. Two experiments were carried out to discover whether progeny of the Type II virus that survived heating for 1 hour at 75°C were more thermoresistant than components of the

TABLE II. Recovery of Poliovirus after Exposure to Various Temperatures for One Hour.

	Temp.	65°C				75°C	85°C
	Exp. No.	1	2	3	6	4	5
Poliovirus type	1			1/15*	0/3†	0/15	0/15
	2	4/10	1/15	"	" †	1/15‡	"
	3			0/15	1/3†	0/15	"

* No. tubes positive/No. tubes inoculated.
† Each tube inoculated with 0.6-1.5 ml instead of 0.1 ml.
‡ Identified by neutralization.

Titer of virus prior to heating: Type 1: $10^{7.3}$ TCD_{50}/ml. Type 2 exp. 1 & 2: $10^{7.3}$ TCD_{50}/ml. Type 2 exp. 3-6: $10^{6.5}$ TCD_{50}/ml. Type 3: $10^{7.3}$ TCD_{50}/ml.

TABLE III. Time-lapse between Inoculation of Heated Poliovirus Type II and First Appearance of Cytopathic Changes.

Exp.	Temp. (°C)	Time heated (min.)	No. cultures with CPE	Interval (days) CPE first observed* 3–5	8–11	13–19	21–27	29	32–35
1	55	10	7	5	1	1			
		20	4	3					
		30	4	1	3				
		60	1	1					
	60	10				1			
	65	10							1†
		30					1		
		60					4		
2	60	10	1		1				
		60	1		1				
	65	10	3		1	1			1
		60	1						1
3	65	60	1		1				
4	75	60	1			1			

* In repeated titrations of unheated virus suspensions CPE was not noted after 6th day following inoculation.

† Questionable CPE on 32nd day.

original unheated stock suspension. A suspension of progeny virus was prepared consisting of fluid from a bottle culture of the first passage in monkey kidney cells. The culture was rinsed 5 times with Hanks' solution before and after inoculation and adsorption of virus. The viral suspension, therefore, probably contained less protein than the unheated suspension which was prepared in cultures that received fewer rinsings. After harvest, the suspension was centrifuged at 2000 rpm for 30 minutes at 4°C. Aliquots were heated at 55°C for various periods together with others of the stock Type II suspension from which it was originally derived. Assay of residual virus in each aliquot was carried out in amnion cell cultures. Results are presented in Fig. 1. It is evident that the progeny of the heated virus was more thermolabile than the parent or parents that had resisted 65°C, since a large proportion was destroyed at 55°C within 10 minutes. The greater overall thermoresistance of progeny virus however, as compared with that of the original unheated stock suspension is equally apparent. Both inactivation curves are inconsistent with a first order reaction.

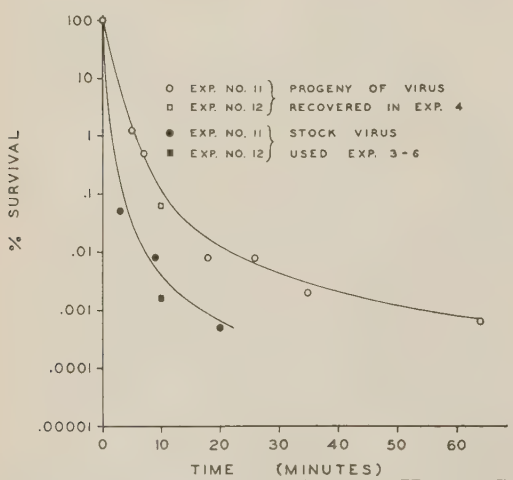


FIG. 1. Inactivation of poliovirus type II at 55°C. Comparison of "heated" and "unheated" virus.

To determine whether this difference in thermostability might be correlated with a difference in plaque morphology, dilute suspensions of progeny and stock viruses were applied to monolayers of amnion cells cultivated in small bottles. Nutrient agar was added. Plaques developed in each which were indistinguishable in respect to shape and size. Other characteristics that might differentiate them such as virulence for animals were not investigated.

Discussion. Two hypotheses may be presented that could account for the presence of these rare, highly thermoresistant forms. The first would regard them as extreme variants in a population in which there is a tendency by many individuals to diverge considerably from the mean thermoresistance. This has been

tacitly accepted in the foregoing portion of the paper. The second hypothesis would envision a primary injury of all units rendering them incapable of initiating infection. Thereafter resynthesis might rarely occur of components derived from different viral particles resulting in production of complete virus. Such a process would be analogous to multiplicity reactivation in bacteriophage. The only datum now available favoring this view is the observed delay in emergence of cytopathic change. At present, therefore, we incline to accept the first and simpler concept which is supported by the demonstrated decrease in rate of inactivation as heating is continued. It would seem, however, that on the basis of our findings (and they should be supplemented by results of experiments with clonal lines of virus) these highly resistant variants do not "breed true" in the sense that their progeny are equally resistant; instead, as one might expect, mean resistance of progeny is increased. This suggests that more than a single gene may determine, in association with environmental phenomena, thermoresistance of the progeny.

Factors to account for the difference between our results and those of previous investigators are not readily apparent. The concentration of extraneous protein in our preparations which might enhance thermostability was as low or lower than that in preparations examined by others. The pH was maintained at neutrality or slightly on the alkaline side where the stabilizing effect of cystine is not critically operative(11). It is possible that the higher concentrations of virus we employed together with replicate tests in a fairly large number of cell cultures of high sensitivity observed over long periods may be responsible for the discrepancy.

At first glance the data presented in Table I appear to suggest that thermoresistance might be somewhat greater at 65° than at 60°C. This paradoxical situation is probably to be attributed to the small number of determinations made. Survival of appreciable quantities of poliovirus under these conditions makes it doubtful whether pasteurization of milk and other food products as now carried out will assure completely inactivation of these agents.

Summary. Suspensions of Polioviruses Types I, II and III in medium 199 derived from infected monkey kidney cell cultures were not completely inactivated following exposure for 1 hour to temperatures of 60° and 65°C. In one instance infectious virus was demonstrated in a suspension of Poliovirus Type II after heating at 75°C for 1 hour. Progeny of virus surviving at this temperature exhibited increased thermoresistance.

1. Paul, J. R., Melnick, J. L., *Poliomyelitis. Diagnostic Procedures for Virus and Rickettsial Diseases,* 2nd ed., Am. Pub. Health Assn., 1956.

2. Howe, H. A., Wilson, J. L., *Poliomyelitis.* Rivers, T. M., Horsfall, F. L., Ed., *Viral and Rickettsial Infections of Man,* 3rd ed., J. B. Lippincott, 1959, 447.

3. Kaplan, A. S., Melnick, J. L., *Am. J. Pub. Health,* 1952, v42, 525.

4. Stanley, N. F., Dorman, D. C., Ponsford, J., Larkin, M., *Austral. J. Exp. Biol. and Med. Sci.,* 1956, v34, 411.

5. ———, *ibid.,* 1956, v34, 297.

6. Youngner, J. S., *J. Immunol.,* 1957, v78, 282.

7. Lwoff, A., *Bact. Rev.,* 1959, v23, 109.

8. Melnick, J. L., Tissue Culture Methods for the Cultivation of Poliomyelitis and Other Viruses. *Diagnostic Procedures for Virus and Rickettsial Diseases,* 2nd ed., Am. Pub. Health Assn., 1956, 97.

9. Frothingham, T. E., PROC. SOC. EXP. BIOL. AND MED., 1959, v100, 505.

10. Katz, S. L., Milovanic, M. V., Enders, J. F., *ibid.,* 1958, v97, 23.

11. Pohjanpelto, P., *Virology,* 1958, v6, 472.

Received April 28, 1960. P.S.E.B.M., 1960, v104.

Role of Intestinal Bacteria in Aromatization of Quinic Acid in Man and Guinea Pig.* (25860)

Ramzi Cotran, Mary I. Kendrick and Edward H. Kass
Mallory Inst. of Pathology, Thorndike Memorial Lab., Second and Fourth (Harvard) Medical Services, Boston City Hospital and Depts. of Pathology and Bacteriology and Immunology, Harvard Medical School

Conversion of non-aromatic cyclic hydrocarbons to aromatic substances by mammals is poorly understood. Aromatization of cyclohexane-carboxylic acid and a small number of related compounds was observed by Friedman(1) and by Bernhard(2,3), and confirmed by Dickens(4). Beer *et al.*(5) demonstrated that slices of liver and kidney, as well as the intact animal, could aromatize several cyclohexanecarboxylic acids. Recently it has been observed that hippuric acid is bacteriostatic to common bacterial pathogens of the urinary tract(6). The major recognized source of urinary hippuric acid is benzoic acid. However, not all of the hippuric acid found in urine can be accounted for on the basis of benzoic acid content of ordinary diets. Quinic acid (1,3,4,5 tetrahydroxy*cyclo*hexanecarboxylic acid), which is present in many fruits and vegetables, has been suggested as a major non-aromatic dietary precursor to hippuric acid(7,8).

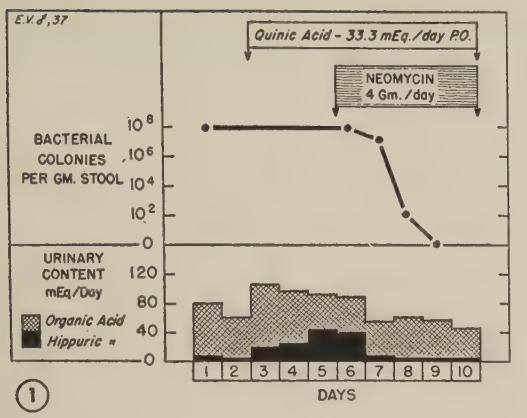
In man, Lauteman(9), Quick(10), Beer *et al.*(6), and Bernhard(11), have demonstrated conversion of ingested quinic acid to hippuric acid. In guinea pigs, Beer *et al.*(5) found little or no conversion of quinic acid to hippuric acid, whereas Bernhard(11) observed that guinea pigs excreted about 60% of ingested quinic acid as benzoic or hippuric acids. In experiments conducted by Beer *et al.*, in which little or no aromatization was observed, quinic acid was given subcutaneously. Davis (12) has demonstrated that coliform bacteria can aromatize quinic acid.

Material and methods. Healthy male volunteers ingested their usual diets. Their urine was refrigerated immediately after collection, and aliquots from 24 hour specimens were stored at –20°C. Stool specimens were obtained at least once daily. Male guinea pigs, weighing approximately 400 g, were maintained in pairs in metabolic cages, on diet of cabbage given *ad lib.* Bacterial multiplication in urine of guinea pigs was inhibited by adding 4 ml of 4N HCl to each container. Quinic acid was obtained from A. D. Mackay Inc. and for human studies was packed in gelatin capsules, each containing 0.5 g. For guinea pigs, quinic acid was dissolved in water, adjusted to pH 7.4 by addition of small amounts of alkali, and administered as single dose of 7 ml by stomach tube or intraperitoneal injection. Neomycin† was given as 0.5 g tablets. Hippuric acid in urine was estimated by paper chromatographic method of Gaffney(13), as modified by Armstrong (14). Organic acids were determined by modification of the method of Van Slyke and Palmer(6). For bacteriologic examination of the stool, aliquots of each specimen were suspended in 10 times their volume of broth, and shaken to make an even suspension. Serial dilutions were then made in nutrient broth, plated in meat infusion agar and colony counts made after aerobic incubation at 37°C for 24 hours. A loopful of each specimen was spread over surface of sheep-blood agar plate for determinative purposes.

Results. Each of 2 volunteers ingested 6 g of quinic acid daily for 3 days, after 2 days of control observation. On fourth day of administration of quinic acid, neomycin was added and given until stool cultures had fallen to 10^3 colonies/g or below. This required 3-4 days in each of the 2 volunteers studied. The third volunteer ingested quinic acid for 3 days, then after an interval of 9 days took quinic acid and neomycin for 4 days. Fig. 1 gives results for one volunteer. Data in the other 2 were comparable. In all

* Supported by grants from Nat. Inst. Health, U.S.P.H.S., and Mass. Heart Assn.

† Kindly supplied by Dr. A. Moriarity, Upjohn Co.



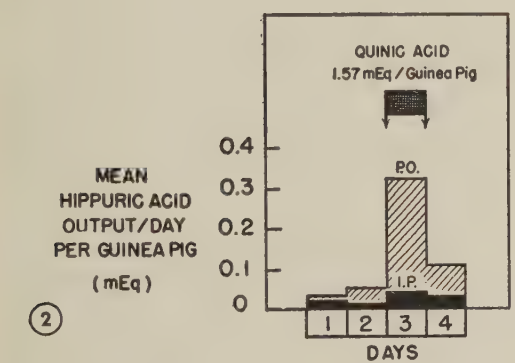


FIG. 1. Effect of neomycin on conversion of quinic to hippuric acid by intestinal flora in man.

FIG. 2. Effect of oral and parenteral administration of quinic acid on hippuric acid excretion.

3 ingestion of quinic acid was followed by recovery of about 70% of administered acid as hippuric acid in urine. Addition of neomycin to the diet led to prompt fall in excretion of hippuric acid to control levels, or to levels below control values, despite continued ingestion of quinic acid.

In the guinea pigs, oral administration of quinic acid also led to prompt excretion of amounts of hippuric acid sufficient to account for 30% of ingested quinic acid (Fig. 2). When quinic acid was given intraperitoneally no significant increase in excretion of hippuric acid was found, but organic acid content of urine rose in correspondence with amount of quinic acid injected.

Discussion. That inhibition of bacterial multiplication in intestinal tract is accompanied by failure of this conversion to occur argues strongly that aromatization is accomplished by the intestinal bacteria. Although the possibility of direct action of neomycin on absorption or aromatization cannot be completely eliminated, it seems unlikely. The malabsorption syndrome occasionalliy reported after administration of neomycin(15) occurs with larger doses, given for longer period, and does not lead to complete absence of absorption from the intestinal tract. The volunteers experienced slight epigastric discomfort but no changes in frequency or character of the stool suggestive of malabsorption. Furthermore, the clear differences in response to intraperitoneal as opposed to oral administration of quinic acid in the guinea pig indicate that only the intestinal bacteria achieve aromatization of quinic acid in this animal species.

Summary and conclusions. When quinic acid is given orally to man and to guinea pigs, it is aromatized and appears in urine as hippuric acid. Neomycin, in doses sufficient to inhibit bacterial multiplication in the intestinal tract, prevents conversion of quinic to hippuric acid in man. When quinic acid is given parenterally to guinea pigs, it is not converted to hippuric acid. It is concluded that aromatization of quinic acid in man and in guinea pig is achieved by intestinal bacteria.

1. Friedman, E., *Hoppe-Seyl Z.,* 1911, v35, 49.

2. Bernhard, K., *ibid.,* 1938, v256, 49.

3. Bernhard, K., Cafleish-Weil, H., *Helvet. chir. acta,* 1945, v28, 1697.

4. Dickens, F., *Nature,* 1947, v159, 839.

5. Beer, C. T., Dickens, F., Pearson, J., *Biochem. J.,* 1951, v48, 222.

6. Bodel, P. T., Cotran, R., Kass, E. H., *J. Lab. Clin. Med.,* 1959, v54, 881.

7. Blatherwick, N. R., Long, M. L., *J. Biol. Chem.,* 1923, v57, 815.

8. Fellers, C. R., Redman, B. C., Parrot, E. M., *J. Nutrition,* 1933, v6, 455.

9. Lauteman, E., *Liebig's Ann. d.Chem.,* 1863, v125, 9.

10. Quick, A. J., *J. Biol. Chem.,* 1931, v92, 65.

11. Bernhard, K., *Helvet. chir. acta,* 1955, v38, 1438.

12. Davis, B. D., Weiss, U., *Arch. exp. Path. u. Pharmakol.,* 1953, v220, 1.

13. Gaffney, G. W., Shreier, K., diFerranti, H, Altman, K., *J. Biol. Chem.,* 1954, v206, 695.

14. Armstrong, M. D., Chao, F. C., Parker, V. J., Wall, P. E., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 675.

15. Jacobson, E., Chodos, R., Falocn, W., *Clin. Res.*, 1959, v7, 33.

Received May 5, 1960. P.S.E.B.M., 1960, v104.

Demonstration of Thiosemicarbazide-Induced Convulsions in Rats with Elevated Brain Levels of γ-Aminobutyric Acid.* (25861)

CLAUDE F. BAXTER AND EUGENE ROBERTS
Dept. of Biochemistry, City of Hope Medical Center, Duarte, Calif.

The brain and spinal cord of vertebrate organisms contain high levels of GABA†(1). Physiological experiments have shown GABA to have inhibitory properties in invertebrate and vertebrate nervous systems(2,3). When TSC was injected into rats, it lowered cerebral levels of GABA probably by preferential inhibition of glutamic acid decarboxylase and increased susceptibility of these animals to convulsions(4). When NH_2OH was injected into rats, cats or monkeys, it elevated cerebral levels of GABA in all 3 species(5) presumably through inhibition of γ-amino-butyric acid-α-ketoglutaric acid transaminase(6). At the same time it was noted that rats were protected against Metrazol-induced convulsions after NH_2OH administration and that in the cat and monkey sensitivity of the cortex to electrical stimulation was decreased(7,8). These and similar observations suggested that certain types of convulsions and resistance to them may be related to levels of GABA in the brain of mammals(9,10). The experiments presented here demonstrate that the physiological effects of TSC and NH_2OH cannot be attributed solely to their effect upon the mechanisms which regulate synthesis and degradation of GABA and that a greatly elevated level of GABA can exist in most brain areas of rats undergoing thiosemicarbazide-induced convulsions.

Method. Twenty-eight male Sprague-Dawley rats weighing 190 to 210 g each, were selected for this experiment and divided into 6 groups. Rats in 5 groups were injected intraperitoneally either with $NH_2OH \cdot HCl$ (75 mg/kg neutralized) or with TSC (20 mg/kg) or with both at varying time intervals as indicated in Table I. Animals injected with $NH_2OH \cdot HCl$ only, were decapitated at 90 to 120 minutes after injection. *All* animals receiving TSC had convulsions within 90 to 135 minutes after injection. Pretreatment with NH_2OH tended to shorten the time period between injection of TSC and onset of convulsions. All TSC-treated animals were sacrificed after showing one maximal tonic extensor convulsion. Nine areas were removed from each brain and analyzed for content of GABA by an enzymatic procedure described previously(5).

Results. TSC alone lowered levels of GABA while NH_2OH alone elevated them in all areas (Table I), in agreement with previous observations(11,5).

In the 3 groups of animals receiving both TSC and NH_2OH, levels of GABA were significantly elevated in most of the areas examined. Several areas showed normal levels, but none were depressed at time of maximal convulsion. In comparing the results of the 3 groups of rats receiving both TSC and NH_2OH, it was found that level of GABA was elevated to a greater extent in the group receiving NH_2OH prior to TSC. This effect was especially noticeable in the colliculi, diencephalon and midbrain tegmentum, areas of brain with the highest levels of glutamic acid decarboxylase (Baxter and Roberts, unpublished).

Discussion. The above results do not ex-

* Supported in part by grants from Nat. Assn. for Mental Health, from Nat. Inst. for Neurol. Dis. and Blindness, U.S.P.H.S.

† Abbreviations: GABA: γ-aminobutyric acid; TSC: thiosemicarbazide; NH_2OH: hydroxylamine.

TABLE I. Influence of NH_2OH and TSC, Alone and in Combination, on GABA Levels in Rat Brain.

Brain areas	GABA levels (mg/100 g of tissue, wet wt)					
	Control* (5)†	NH_2OH only* (3)	TSC only (4)	TSC ½ hr after NH_2OH (4)	TSC + NH_2OH together (5)	TSC ½ hr before NH_2OH (5)
Cortex	25‡ (24-27)	49 (45-54)	18 (16-20)	48 (42-57)	41 (36-45)	37 (28-47)
Medulla	25 (23-26)	33 (28-38)	17 (16-18)	34 (28-44)	29 (27-32)	26 (22-29)
Pons	27 (25-28)	42 (38-46)	18 (17-19)	42 (34-53)		31 (27-37)
Cerebellum	27 (25-30)	37 (35-38)	20 (19-23)	44 (38-51)	37 (30-49)	38 (29-43)
Hippocampus	31 (28-35)	41 (38-44)	20 (18-22)	42 (39-44)	39 (36-43)	39 (27-50)
Caudate nucleus	31 (29-33)	46 (42-52)	22 (20-24)	45 (31-54)	41 (33-55)	43 (29-51)
Olfactory lobes	48 (38-55)	61 (44-78)	43 (37-51)	65 (57-78)	62 (52-75)	61 (50-77)
Diencephalon	53 (46-60)	71 (62-75)	32 (27-36)	66 (54-88)	57 (52-63)	56 (42-71)
Colliculi	56 (52-60)	85 (77-95)	33 (31-35)	73 (60-94)	58 (54-65)	53 (38-66)

Animals receiving NH_2OH only, were sacrificed 90 to 120 min. after inj. All animals receiving TSC, alone or in combination with NH_2OH, were sacrificed at time of first maximal tonic convulsion.

* For more extensive series showing similar results see Baxter and Roberts(5).

† Figures in parentheses indicate No. of rats.

‡ Avg for group; figures in parentheses indicate range.

clude the possibility that a decrease occurred in level of GABA in specific small areas of brain. However, they indicate clearly that an overall depression of GABA levels is not a necessary requirement for TSC-induced convulsions. This conclusion is in keeping with recent physiological findings by Adey *et al.* (12) and by Terzuolo *et al.*(13).

Although in the past attention has been focused upon the function of TSC and NH_2OH as carbonyl trapping agents which affect Vit. B_6 dependent enzyme systems, these agents may well have additional effects upon various cellular constituents including enzymes which do not require pyridoxal phosphate as cofactor. TSC may exert an effect upon neuronal membranes or prevent formation of some physiologically active derivative of GABA. These and other possibilities are under investigation.

Summary. In confirmation of biochemical data for whole brain, injection of thiosemicarbazide produced convulsive seizures in rats concomitantly with decreases in levels of γ-aminobutyric acid in 9 areas of the brain. However, rats injected with both hydroxylamine and thiosemicarbazide had convulsive seizures when levels of γ-aminobutyric acid were either elevated or normal in different areas of brain. Seizures produced by administration of thiosemicarbazide are not related to a generally decreased level of γ-aminobutyric acid in the brain.

1. Roberts, E., Lowe, I. P., Guth, L., Jelinek, B., *J Exp. Zool.,* 1958, v138, 313.

2. Kuffler, S. N., Edwards, C., *J. Neurophysiol.,* 1958, v21, 589.

3. Dasgupta, S. R., Killam, E. K., Killam, K. F., *J. Pharmacol. Exp. Ther.,* 1958, v122, 16A.

4. Killam, K. F., Bain, J. A., *ibid.,* 1957, v119, 255.

5. Baxter, C. F., Roberts, E., PROC. SOC. EXP. BIOL. AND MED., 1959, v101, 811.

6. ———, *Fed. Proc.,* 1960, v19, 1.

7. Eidelberg, E., Baxter, C. F., Roberts, E., Saldias, C. A., French, J. D., PROC. SOC. EXP. BIOL. AND MED., 1959, v101, 815.

8. Roberts, E., Baxter, C. F., Eidelberg, E., *Function and Structure of the Cerebral Cortex,* Elsevier Publishing Co., Amsterdam, 1960, in press.

9. Killam, K. F., Dasgupta, S. R., Killam, E. K., *Inhibition in the Nervous System and γ-Aminobutyric Acid,* Pergamon Press, London, 1960, p302.

10. Elliott, K. A. C., Jasper, H. H., *Physiol. Rev.,* 1959, v39, 383.

11. Killam, K. F., *J. Pharmacol. Exp. Ther.,* 1957, v119, 263.

12. Adey, W. R., Dunlop, C. W., Killam, K. F., Brazier, M. A., *Inhibition in the Nervous System and γ-Aminobutyric Acid,* Pergamon Press, London, 1960, p317.

13. Terzuolo, C. A., Sigg, B., Killam, K. F., *ibid.,* p336.

Received May 9, 1960. P.S.E.B.M., 1960, v104.

Production of Renal Disease in the White Mouse with Streptococcal Infection.* (25862)

JOHN T. SHARP (Introduced by E. B. Taft)
Massachusetts General Hospital, Boston

In man group A streptococcal infection usually precedes onset of acute glomerulonephritis and frequently precedes an exacerbation of chronic nephritis. Several investigators have reported renal disease in animals infected with streptococci or inoculated with streptococcal products, but none of these systems contributed to our understanding of human nephritis and the search for suitable model continues. In this laboratory a new experimental renal disease has been observed in mice inoculated with living streptococci. The circumstances for production of this experimental disease and the extent of the process, characterized by albuminuria, hypoalbuminemia, generalized edema, and histological changes are described.

Materials and methods. Infections were produced with streptococcal strains D58 (type 3), D58/XXD2, GL8 (type 19), and ADA (type 14). These bacteria were originally isolated in other laboratories from humans without renal disease and maintained for several years by repeated subculture without animal passage except for the D58/XXD2 strain which was passed in one mouse. *Staphylococcus aureus* strains 80 and 81, originally isolated from carbuncles, were obtained from a phage-typing laboratory. *S. aureus* 3859 and *E. coli* 597 were recently isolated in hospital laboratory from septic processes. The animals used were adult, male, white mice of Webster strain from Harvard Medical School colony. They usually weighed 17 to 23 g at beginning of experiment. Injections were made subcutaneously in the dorsal aspect of either hindquarter. Inocula were prepared by suspending the centrifuged sediment of 18-22 hour broth cultures in fresh broth or sterile, light mineral oil. Concentrations of inocula were expressed either as number of organisms/animal injected, as determined by optical density at 640 mμ, or as concentration relative to original culture. With the latter method an inoculum designated 5:1 refers to the sediment from 5 ml of culture suspended in 1 ml of new medium. Paper electrophoresis of serum was performed with veronal buffer, pH 8.6 and 0.1 μ. Electrophoresis of urinary proteins was performed in 4 buffers: veronal, pH 8.6, phosphate, pH 7.0, and acetate at pH 5.5 and 4.0.

Results. Observations with streptococcal infections. Animals injected with 10^8 D58 or D58/XXD2 organisms developed marked swelling of inoculated quarter in 1-2 days. Local swelling usually persisted for duration of experiment. Most animals showed weight loss during first 2 to 4 days of infection. Thereafter many showed rapid weight gain, culminating in recognizable abdominal swelling between 5th and 10th day. Of 215 animals inoculated with large numbers of the D58 strain, 123 developed ascites as demonstrated by paracentesis or at autopsy (Table I). At times edema was extensive with weight gain of ⅓ or more of preinoculation body weight.

TABLE I. Incidence of Ascites.

Inoculations	Dose range	No. animals inj.	No. with ascites*
Streptococcus			
D58	.5 ml 4:1 to 33:1	73	40
"	$1.2\text{-}9.5 \times 10^8$	127	83
"	$2.0\text{-}8.2 \times 10^7$	15	0
GL8	.5 ml 33:1	5	2
ADA	.5 ml 33:1	9	1
S. aureus 3859, 80, or 81	$7\text{-}35 \times 10^9$	70	0
E. coli 597	$1.8\text{-}1.9 \times 10^{10}$	20	0
Oil, broth, and heat killed streptococci		33	0

* Ascites proven by autopsy or paracentesis. All animals had 1 or more attempted paracentesis between 5 and 14 days after initiating infection.

* This is publication No. 276 of the Robert W. Lovett Memorial Unit for Study of Crippling Disease. Expenses of this investigation have been defrayed by grants from Commonwealth Fund, Nat. Inst. of Arthritis and Metab. Dis., Mass. Heart Assn., and Eli Lilly Co.

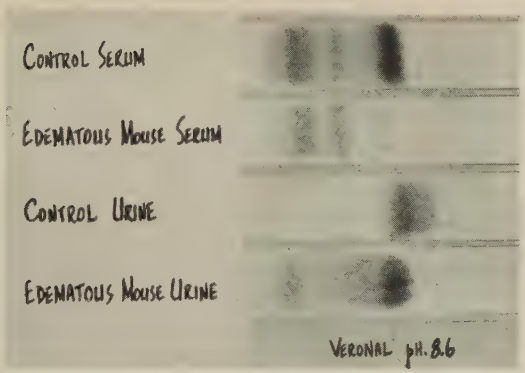


FIG. 1.

Paper electrophoresis demonstrated a decrease in albumin concentration in sera of all edematous animals studied. In 4 groups of edematous animals, 12 were successfully bled. Their average albumin concentration was 12.1%, range 2.1-19.5%. Six control animals had an albumin concentration of 40.0% with range of 20.7-56.4% (Fig. 1).

Since moderately large amounts of protein were found in urine of normal mice, partial characterization of urinary proteins from normal and edematous animals was undertaken to determine whether qualitative differences existed between the 2 groups. Electrophoresis at pH 8.6, 5.5, and 4.0 demonstrated 2 components in normal urinary protein and both moved faster than serum albumin. At all pH values urinary protein of edematous animals had mobilities comparable to serum proteins, with largest amount of material corresponding to albumin (Fig. 1). Ultracentrifugation also distinguished between urines of the 2 groups. Protein of normal animals had a sedimentation constant of 2.4, whereas the major component of edematous animals' urine and mouse albumin had constants of 4.6 to 5.0.

At autopsy kidneys of all edematous animals were pale, as were those of some animals without edema. Necrosis of proximal convoluted tubules and hyaline casts in the collecting tubules were seen by light microscopy during 2 to 14 days after initiating infection. In many instances tubular necrosis was so extensive that the tubules were denuded of cellular elements. This tubular lesion was believed to account for the gross pallor. Electron microscopic examination of glomeruli from kidneys of edematous animals at 13 days revealed fusion of epithelial foot processes. In animals sacrificed 30-40 days after inoculation thickening, fusion, and irregular dilatation of glomerular capillaries were observed by light microscopy. Evidence of generalized infection was usually absent. In several experiments lung weights of animals with generalized edema were comparable to those of controls.

Studies of a few animals infected with GL8 and ADA strains of streptococci established that a similar experimental disease may be associated with infection by strains other than the D58 (Table I).

Other animals were injected with heat killed streptococci, sterile broth, or sterile oil, and none developed edema. Serum and urinary proteins were normal by electrophoresis in several instances and microscopic changes were not found in the kidneys. Penicillin treatment of infected animals prevented occurrence of edema when initiated at time of injection of streptococci but did not prevent its development when given 24 hours or more after the bacteria.

Cultures made from kidneys of infected animals were usually sterile in absence of blood stream invasion (Table II).

Observations with other infections. Twenty animals infected with approximately 10^{10} *E. coli* cells and 70 mice infected with 10^9 or 10^{10} *S. aureus* organisms never developed ascites or edema. Concentration of albumin averaged 29.4% of total protein with range of 13 to 38% in sera of 18 of these mice. Urinary proteins in 6 pooled collections from 15 staphylococcus infected animals and in 4 collections from 13 *E. coli* infected animals

TABLE II. Cultural Data.

Infecting organism	No. with edema cultured	β-hemolytic streptococci recovered			
		Blood		Kidneys	
		No. pos.	No. done	No. pos.	No. done
D58 or D58/XXD2	26	0	41	1*	39
GL8	2	3	3	3	3
ADA	1	0	5	1	5

* Blood from this animal was not cultured.

were also normal by electrophoresis. Microscopic examination of representative kidneys from animals infected with these organisms were normal.

Discussion. Several investigators reported histological changes in kidneys of animals infected with streptococci or inoculated with streptococcal products(1-8). However association of albuminuria, hypoalbuminemia, and generalized edema with experimental streptococcal infection represents a new observation and suggests that group A streptococcal infection in these animals induced a nephrotic syndrome. The finding of an abnormality in the glomerular ultrastructure similar to that seen in spontaneously occurring nephrosis and nephrosis induced by other agents supports such an interpretation(9-13). However extensive tubular necrosis is not a feature of the nephrotic syndrome and the role of the tubular lesion in this experimental disease must be considered.

Renal shutdown as a mechanism for production of edema in these animals is unlikely since fluid was not forced, lung weights were not increased in edematous animals, and hypoalbuminemia was present in all edematous animals whose sera were studied electrophoretically. The possibility that a decrease in tubular reabsorption of protein was responsible for serum protein changes is also unlikely(14,15). Based on studies of albumin clearance 3 groups of investigators calculated that maximum possible reabsorption of protein by the tubules was small compared with that appearing in urine in pathological states (16,17,18). It appears reasonable to assume that the observed proteinuria is not the result of a tubular lesion but reflects increased glomerular permeability. The majority of evidence therefore favors the interpretation that tubular necrosis is not essential to the clinical picture but is either superimposed on or concomitant with a glomerular lesion responsible for an otherwise clear cut nephrotic syndrome.

In addition to characterizing clinical and pathological features of the experimental disease these studies suggest that growth of injected streptococci was necessary for induction of the disease. Thus inoculation of heat killed organisms failed to induce the experimental disease and penicillin treatment initiated at time of infecting inoculation prevented its development. The further observation that infection with large numbers of staphylococci or coliform organisms failed to induce renal changes suggests that properties of the infecting organism determine the occurrence of this nephropathy.

Summary. Development of albuminuria, hypoalbuminemia, and generalized edema was observed 5-10 days after initiating streptococcal infection in the male Webster white mouse. Animals with this clinical picture had pale kidneys which showed tubular necrosis by light microscopy, and glomerular changes by electron microscopy. Animals surviving 4 to 6 weeks also showed glomerular changes by light microscopy. It is proposed that these observations may be interpreted as production of the nephrotic syndrome by streptococcal infection with a superimposed or concomitant tubular lesion.

1. Bell, E. T., Clawson, B. J., *Am. J. Path.,* 1931, v7, 57.

2. Duval, C. W., Hibbard, R. J., *J. Exp. Med.,* 1926, v44, 567.

3. Helmholz, H. F., *Arch. Path.,* 1932, v13, 592.

4. Lillehei, C. W., Bobb, J. R. R., Wargo, D., Visscher, M. B., Shaffer, J. M., Bell, E. T., *Circulation,* 1951, v4, 587.

5. Reed, R. W., Matheson, B. H., *J. Inf. Dis.,* 1954, v95, 191.

6. Kelly, D. K., Winn, J. F., *Science,* 1958, v127, 1337.

7. Matheson, B. H., Reed, R. W., *J. Inf. Dis.,* 1959, v104, 213.

8. Cavelti, P. A., Cavelti, E. S., *Arch. Path.,* 1945, v40, 158.

9. Farquhar, M. G., Vernier, R. L., Good, R. A., *J. Exp. Med.,* 1957, v106, 649.

10. ———, *Am. J. Path.,* 1957, v33, 791.

11. Vernier, R. L., Papermaster, B. W., Good, R. A., *J. Exp. Med.,* 1959, v109, 115.

12. Piel, C., Proc. of 10th Annual Conference on Nephrotic Syndrome (edited by J. Metcoff), 1958.

13. Feldman, J., *ibid.*

14. Walker, A. M., Oliver, J., *Am. J. Physiol.,* 1941, v134, 562.

15. Sellers, A. L., Griggs, N., Marmorston, J., Goodman, H., *J. Exp. Med.,* 1954, v100, 1.

16. Chinard, F. P., Lauson, H. D., Eder, H. A.,

Greif, R. L., Hiller, A., *J. Clin. Invest.*, 1954, v33, 621.

17. Hardwicke, J., Squire, J. R., *Clin. Sci.*, 1955, v14, 509.

18. Gregoire, F., Malmendier, C., Lambert, P. P., *Am. J. Med.*, 1958, v25, 516.

Received May 10, 1960. P.S.E.B.M., 1960, v104.

Relative Effectiveness of B_{12} and Dimethylbenzimidazole B_{12}-Coenzyme in Thymine Methyl Biosynthesis.* (25863)

James S. Dinning

Dept. of Biochemistry, University of Arkansas School of Medicine, Little Rock

We have recently reported that Vit. B_{12} stimulates conversion of formate to 5-methyl group of thymine by chick bone marrow cells (1). Further studies have indicated that the vitamin functions in reduction of one-carbon compounds between formate and formaldehyde levels of oxidation(2,3). Barker *et al.*(4) reported isolation of coenzyme forms of B_{12} which catalyze conversion of glutamate to β-methylasparate by certain microorganisms. In this system B_{12} is inactive. It appeared desirable to determine effectiveness of the coenzyme form of B_{12} in stimulating conversion of formate to thymine methyl.

Methods. Handling of chicks, preparation of bone marrow cell suspensions, details of incubation and formation of the acetol osazone from thymine were the same as previously described(1). Bone marrow cells from B_{12}-deficient chicks were incubated with formate-C^{14} and deoxyuridine and various concentrations of Vit. B_{12} or 5,6 dimethyl-benzimidazole B_{12}-coenzyme(4). The results are reported as per cent increase in conversion of formate-C^{14} to thymine as a result of addition of B_{12} or the coenzyme.

Results. Each result given in Table I is the average of at least 2 experiments. It is quite clear that the coenzyme form of B_{12} was less effective than the crystalline vitamin in stimulating conversion of formate to the 5 methyl group of thymine. This observation is in agreement with other reports indicating that the coenzyme form of B_{12} does not stimulate methionine biosynthesis under conditions in which the vitamin is effective(5,6). It appears that Vit. B_{12} has at least 2 distinct metabolic roles, one in reduction of one carbon compounds and the other in biochemical rearrangements catalyzed by coenzymes first described by Barker *et al.*(4).

TABLE I. Influence of Vit. B_{12} and 5, 6 Dimethylbenzimidazole B_{12} Coenzyme (DMBC) on Conversion of Formate C^{14} to Thymine Methyl by B_{12}-deficient Chick Bone Marrow Cells. (Results expressed as % increase in conversion of formate to thymine methyl.)

mγ/ml	B_{12}	DMBC
15	35	9
150	66	34
1500	90	43
3750		47

Summary. Vit. B_{12} was more effective in stimulating conversion of formate to thymine methyl by chick bone marrow cells than was 5,6 dimethylbenzimidazole B_{12}-coenzyme.

* This work was supported by grant from U.S.P.H.S.

1. Dinning, J. S., Young, R. S., *J. Biol. Chem.*, 1959, v234, 1199.

2. ———, *ibid.*, 1959, v234, 3241.

3. Dinning, J. S., Hogan, R., *Fed. Proc.*, 1960, v19, 418.

4. Weissbach, H., Toohey, J. I., Barker, H. A., *Proc. Nat. Acad. Sci.*, U. S., 1959, v45, 521.

5. Kisliuk, R. L., *Fed. Proc.*, 1960, v19, 416.

6. Takeyama, S., Buchanan, J. M., *ibid.*, 1960, v19, 417.

Received May 13, 1960. P.S.E.B.M., 1960, v104.

LH-Releasing Activity in Hypothalamic Extracts. (25864)

S. M. McCann, S. Taleisnik* and H. M. Friedman†
(Introduced by John R. Brobeck)
Dept. of Physiology, School of Medicine,‡ University of Pennsylvania, Philadelphia

Release of luteinizing hormone (LH) from the pituitary appears to be under hypothalamic neurohumoral control(1,2). Since convincing evidence is now available indicating that a corticotrophin-releasing factor and vasopressin can release adrenocorticotrophin from the pituitary(3-6), it seems of interest to examine hypothalamic and median eminence extracts to determine if a substance originating in these structures may regulate release of LH. Ovarian ascorbic acid depletion (OAAD) from heavily luteinized ovaries of suitably prepared immature rats has been shown by Parlow(7) to be an extremely sensitive and specific assay for LH, a finding that we have confirmed(2). In the present study this assay has been used to assess LH-releasing activity of hypothalamic extracts.

Methods. Assay animals were prepared by the following technic. Female rats, 26 days old, of the Wistar or Sherman strain were injected subcutaneously (sc.), first, with 75 International Units (IU) of pregnant mare's serum gonadotrophin (Equinex),§ and 58 to 67 hours later with 30 IU of human chorionic gonadotrophin. Five to 7 days later, under ether anesthesia the left ovary was removed for analysis of its ascorbic acid concentration, and the substance to be assayed was injected intravenously (IV) during one minute. One hour after removal of the first ovary, the right ovary was similarly removed and analyzed for its ascorbic acid concentration by the method of Mindlin and Butler(8). Results were expressed as percentage depletion (decrease) in ovarian ascorbic acid of the second ovary as compared to the first gland. Extracts of the stalk-median eminence (SME) region from adult male rats, normal or hypophysectomized (hypox.),‖ were made by grinding this tissue and adjacent ventral hypothalamus in 0.5 ml of 0.1 N HCl with sand and a stirring rod. Ten or more SME's were pooled, and the volume adjusted with 0.1 N HCl so that quantity to be injected into each assay rat was contained in 1 ml of the mixture. The diluted extract was then centrifuged at 3000 rpm for 15 minutes, and the supernatant assayed as described above. Extracts of rat cerebral cortex were prepared and assayed similarly. Hypox. assay rats were operated upon by the parapharyngeal approach and immediately used for assay as described above.

Results. The results (Table I) indicate that unilateral ovariectomy alone failed to elicit a significant OAAD. When an extract prepared from the SME region of 2 hypox. or normal (Table II) rats was injected into each assay rat, a highly significant OAAD resulted ($P<0.001$). On the contrary, an extract of rat cerebral cortex equivalent in wet weight to that from SME failed to elicit a significant OAAD.

A number of pharmacologically active agents known to exist in hypothalamic tissue was assayed to determine if the OAAD induced by the hypothalamic extract could be accounted for by any of these compounds. Epinephrine and Substance P were both inactive in these assay rats. Histamine and serotonin, on the other hand, in doses estimated to be about 1000 times greater than the amount expected to be present in the extracts of SME(9,10), evoked small but significant OAAD's which were much smaller ($P<0.001$) than that obtained with SME extract. Seventy mU of a commercial extract of vasopressin (Pitressin), corresponding to the quantity of vasopressin previously shown

† Supported by grant from Nat. Science Fn.

* Postdoctoral Fellow of Nat. Inst. Health.

‡ Supported by N.I.H. grant and by Air Force grant.

§ Kindly supplied by Dr. John B. Jewell, Ayerst Labs., N. Y.

‖ Obtained from Hormone Assay Labs., Chicago and used 24 hours post-hypox.

TABLE I. Ovarian Ascorbic Acid Depletion Induced by Hypothalamic Extracts and Various Pharmacological Agents.

Treatment	Ovarian ascorbic acid depletion (OAAD)	P *vs* control
Unilateral ovariectomy (control)	1 ± 1.8(12)*	
Unilateral ovariectomy plus:		
A. Extract stalk-median eminence, hypox. rat, 2 SME's	22 ± 1 (6)	<.001
B. Extract rat cerebral cortex	1 ± 3 (5)	ns†
C. Serotonin creatinine SO_4, 25 μg	8 ± 1.9 (5)	=.05
D. Histamine, .3 mg	9 ± 0.9 (5)	<.05
E. Epinephrine, 2 μg	4 ± 3 (5)	ns
F. Substance P, 1 mg	2 ± 2 (5)	"
G. Pitressin, 70 mU	8 ± 1 (4)	>.05
H. Pitocin, 70 mU	7 ± 3 (6)	ns
I. Pitressin, 70 mU + Pitocin, 15 mU	6 ± 1 (5)	"

* Mean ± S.E. of mean (No. of rats/group).
† ns = no significant difference from control.

to be present in 2 hypox. rat SME's(6), induced an OAAD of borderline significance that was much less than that obtained with SME extract ($P<0.001$). Seventy mU of oxytocin (Pitocin) did not produce a significant OAAD and was not able to potentiate the activity of Pitressin when injected at a dose equivalent to that expected to be present in the injected extract(11).

Because the extract of SME might have contained LH, it was important to assay it in hypox. rats. These were used immediately after hypophysectomy because we found that if we used the rats 18-24 hours post-hypox., there was a very low ovarian ascorbic acid and decreased sensitivity to LH. Even during the one hour interval following removal of the first ovary a small decrease in ovarian ascorbic acid occurred (Table II).

The data in Table II show that the OAAD induced by LH or Pitressin was the same in both normal and hypox. rats. Two or 4 SME's from normal donor rats induced a small OAAD in the hypox. rats, significantly greater than the control value at the higher dose of 4 SME's; however, a significantly greater OAAD was obtained in normal rats at both the 2 and 4 SME dose than occurred in hypox. rats.

Discussion. The results indicate that rat SME or adjacent hypothalamic tissue contains substances which can deplete ovarian ascorbic acid. This is not a non-specific effect, nor can the activity of SME be explained by its content of certain known hypothalamic constituents.

The results with vasopressin and oxytocin are of particular interest since Martini *et al.* (12) have reported a gonadotrophin-releasing action of Pitocin and Pitressin in the rabbit. In contrast in the present experiments all activity of Pitressin was present in the absence of the hypophysis suggesting a direct action of this substance on the ovary.

Since SME extract evoked a greater OAAD in the normal than in the hypox. rat, a release of LH from the hypophysis of the normal assay animals appears to have occurred. The nature of the substance(s) responsible for this action remains to be determined. Further experiments are also required to ascertain if this substance, tentatively designated

TABLE II. Comparison of Effect of LH, Pitressin, and Hypothalamic Extract in Normal and "Acutely" Hypophysectomized Rats.

Treatment	Ovarian ascorbic acid depletion (OAAD) Normal	Hypophysectomized	P (hypox. *vs* normal)
Control (unilateral ovariectomy)	1.3± 1.8 (12)*	8.2± 2.9 (10)	=.05
Unilateral ovariectomy plus:			
A. LH, .2 μg	20 ± 2.3 (10)	17 ± 2.7 (8)	ns†
1.0 "	30 ± 2.5 (10)	29 ± 2.1 (8)	"
B. Pitressin, 0.5 U	21 ± 3.2 (9)	24 ± 2.4 (10)	"
C. Rat SME extract—2 SME's	18 ± 1.5 (14)	11 ± 1.4 (14)	<.005
4 "	22 ± 2.0 (9)	16 ± 1.9 (11)‡	<.05

* Mean ± S.E. of mean (No. of rats/group).
† ns = no significant difference from control.
‡ P <.05 when compared to response to uni-ovariectomy alone in the hypox. rat.

LH-releasing factor, acts directly on the adenohypophysis.

The residual OAAD produced by SME extract in the hypox. rat is presumably due to the presence of LH or vasopressin or a combination of the 2 in the extracts.

Since the difference between the OAAD in normal and hypophysectomized assay rats injected with SME extract was not large, it would appear that the extracts caused release of only a small amount of LH from the hypophysis. As we have shown that content of LH in the pituitary of these immature assay animals is very low, the amount of LH released by the extract is probably a very significant percentage of this total. Their low hypophysial LH content may constitute a major disadvantage to use of these immature rats for assay of LH-releasing activity. Nevertheless, the data show that a significant release of LH was produced by SME extracts in these assay animals.

Summary. Acid extracts of rat SME tissue evoked OAAD in immature rats pre-treated with gonadotrophins. Part of the activity in the extracts could be accounted for by their content of LH or vasopressin or both, whereas the remaining activity appeared to be due to release of LH from pituitary of assay rats. The substance(s) responsible for LH-releasing activity of extracts has been called LH-releasing factor. The nature of this material is unknown, but it appears to differ from histamine, serotonin, substance P, epinephrine, and vasopressin or oxytocin.

The technical assistance of Maria Smith is gratefully acknowledged.

1. Harris, G. W., *The Neural Control of the Pituitary Gland,* Arnold, London, 1955.

2. McCann, S. M., Taleisnik, S., Friedman, H. M., *Fed. Proc.,* 1960, v19, 292.

3. Saffran, M., Schally, A. V., Benfey, B. G., *Endocrinol.,* 1955, v57, 439.

4. Guillemin, R., Hearn, W. R., Cheek, W. R., Householder, D. E., *ibid.,* 1957, v60, 488.

5. Royce, P. C., Sayers, G., Proc. Soc. Exp. Biol. and Med., 1960, v103, 1.

6. McCann, S. M., Haberland, P., *ibid.,* 1959, v102, 319.

7. Parlow, A. F., *Fed. Proc.,* 1958, v17, 402.

8. Mindlin, R. L., Butler, A. W., *J. Biol. Chem.,* 1937, v122, 673.

9. Harris, G. W., Jacobsohn, D., Kahlson, G., *Ciba Foundation Colloquia (Endocrinology IV),* 1952, 186.

10. Udenfriend, S., Shore, P. A., Bogdanski, D. F., Weissback, H., Brodie, B. B., *Recent Prog. Hormone Research,* 1957, v13, 1.

11. Van Dyke, H. B., Adamsons, K., Engel, S. L., in *The Neurohypophysis,* ed. Heller, H., 1957, 67.

12. Martini, L., Mira, L., Pecile, A., Saito, S., *J. Endocrin.,* 1959, v18, 245.

Received May 13, 1960. P.S.E.B.M., 1960, v104.

Crotalaria spectabilis Toxicity in Chickens.* (25865)

J. R. Allen, G. R. Childs† and W. W. Cravens†
Dept. of Pathology, University of Wisconsin School of Medicine, Madison

Crotalaria spectabilis, commonly known as rattlebox, is a leguminous plant grown throughout the South as a cover crop on sandy soils. It grows wild along fence rows, in uncultivated fields and occasionally in row crops after cultivation has been completed. The plant, as well as its seed, is toxic to all farm animals. The toxic principle in *Crotalaria spectabilis* has been isolated and identified as monocrotaline(1,2). Neither lethal dose nor tissue changes associated with its consumption have been clearly defined in chickens(7). Toxicity studies of *Crotalaria spectabilis* for poultry were undertaken after numerous deaths in several large laying flocks that consumed a ration containing an unknown quantity of this legume. Examination of dead and morbid birds invariably revealed ascites and hemorrhage in the liver. It was

* This investigation was supported in part by a grant from Nat. Heart Inst., P.H.S.

† McMillen Feed Mills (Research), Decatur, Ind.

TABLE I. Response of Chicks to Different Concentrations of Crotalaria in the Diet.

Conc. of Crotalaria in diet (%)	No. of chicks that died	Time interval for death (days)	Wt at 5 wk (g)
.0	0/10		596
.05	"		443
.1	2/10*	23-28	312
.2	3/10	18-32	211
.3	9/10	15-35	178
.0	0/10		
.5	10/10	7-18	
1.0	"	5-11	
3.0	"	5-8	
5.0	"	4-7	

* 2/10 signifies that 2 out of 10 birds died within specified interval.

also common to find ruptured liver with hemorrhage into the abdominal cavity of such chickens.

Methods. Fifty Vantress-White Rock sexed broiler chicks were weighed at one day of age and placed on rations containing from 0.5% to 5.0% *Crotalaris spectabilis* seed in a complete commercially prepared broiler ration.† All the test birds died within 19 days. In a second study the level of *Crotalaria spectabilis* was reduced to levels ranging from 0.05% to 0.3%. Surviving birds in the second trial were weighed again at 5 weeks of age. Response of the birds to different concentrations of *Crotalaria spectabilis* in various assays is shown in Table I. Autopsies were performed on all birds that died as well as survivors. Tissue from the heart, liver, kidney, spleen and bone marrow was fixed in 10% formalin, embedded in paraffin and sections cut at 6 μ and stained with hematoxylin and eosin.

Pathology. All levels of *Crotalaria spectabilis* seed produced reduction in growth. As the level was increased, growth retardation was more evident. At concentrations in excess of 0.3% all birds died. Ascites was present in all birds except those in the 2 control rations. Ascitic fluid varied directly with amount of seed fed. Thirty to 75 ml of peritoneal transudate was frequently aspirated from 3 to 5 week old birds. Total protein in this transudate ranged from 0.8 to 2.4 g %. A marked reduction in size of the liver was a constant finding irrespective of concentration of Crotalaria fed. Birds receiving higher concentrations developed these lesions within a shorter period of time. It was not uncommon to find livers less than ⅓ the size of control birds (Fig. 1). These light tan livers were frequently covered by coagulated transudate. Occasionally in chickens fed lower levels (0.1-0.2%) there was a rupture of the liver capsule with hemorrhage into the abdominal cavity. There was also minimal fibrous hyperplasia of the liver capsule. It was not uncommon to find petechial or ecchymotic hemorrhages in liver and lungs. Focal hemorrhages were also seen in the pericardium of some birds. Bone marrow in the test birds was normal.

Discussion. Initially the causative factor responsible for numerous deaths in several large laying flocks was not known. Tentative considerations were toxic fat, excessive salt in the ration and creosote toxicity resulting from disinfectants. These were excluded on the basis of feeding trials and chemical analy-

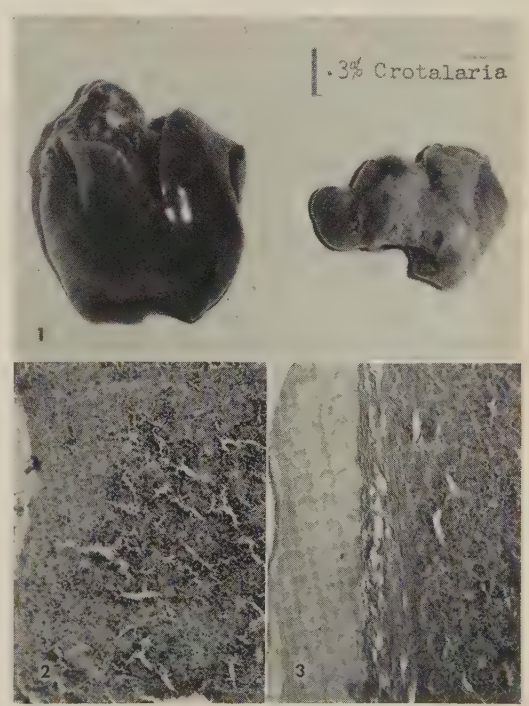


FIG. 1. Differences in size of liver of bird fed 0.3% Crotalaria when compared to control.

FIG. 2. Hemorrhage within liver parenchyma. H & E stain; ×75.

FIG. 3. Coagulated transudate over liver capsule. H & E stain; ×75.

sis of the feed. Inspection of the feed revealed seeds resembling *Crotalaria spectabilis*. Thereafter, inclusion of such seeds in test rations produced lesions similar to those observed in the field.

Crotalaria depresses egg production and causes weight loss in laying birds. Crotalaria is known to produce firm and cirrhotic livers in horses(3,4,5). In chickens that died in these acute toxicity studies cirrhosis was not observed. Cattle consuming Crotalaria seed or plant have a loss of appetite, tenesmus and blood in the feces. Pigs eating Crotalaria develop hemorrhage into the serous membranes, anemia, enteritis, and jaundice(6). No anemia, enteritis, or jaundice was observed in the chickens in these assays.

Summary. Concentrations of 0.05% to 5% *Crotalaria spectabilis* seed were fed to chicks. All concentrations of the legume were shown to be toxic. Concentrations in excess of 0.3% produced death in all birds within 18 days. When less than 0.2% was fed there was a marked reduction in weight gain. In birds which died, hemorrhage was frequently observed in liver, lungs and the pericardium. Atrophy of liver and ascites were constantly encountered in test birds after death.

1. Adams, R., Rogers, E. F., *J. Am. Chem. Soc.*, 1939, v61, 2815.

2. Neal, W. M., Rusoff, L. L., Ahmann, C. F., *ibid.*, 1935, v57, 2560.

3. Gibbons, W. J., Durr, E. H., Cox, S. A., *North Am. Vet.*, 1953, v34, 556.

4. Piercy, P. L., Rusoff, L. L., *J.A.V.M.A.*, 1946, v108, 69.

5. Ritchey, G. E., McKee, R., Becker, R. B., Neal, W. M., Arnold, P. T., *Univ. of Florida Agri. Exp. Station Bull.*, 1941, 361.

6. Berry, D. M., Bras, G., *North Am. Vet.*, 1957, v38, 323.

7. Schmittle, S. C., Richey, D. J., Tumlin, J. T., 48th Meeting Poultry Science Assn., Ames, Iowa, Aug. 1959.

Received May 16, 1960. P.S.E.B.M., 1960, v104.

Tolerance of Chickens for Barium. (25866)

DEWEY JOHNSON, JR., ARNON L. MEHRING, JR., AND HARRY W. TITUS
Limestone Products Corp. of America, Newton, N. J.

Barium carbonate has been used for poisoning rats for many years. Several terms have been used in the literature to describe levels of barium that are toxic to animals. Lethal dose and fatal dose have been used in European literature, and LD_{50} dose in recent American literature. Hüter(1) considered the lethal dose of barium carbonate for rats to be 1500 mg/kg of live weight. Dieke(2) found that LD_{50} of barium carbonate for wild rats was 1480 mg/kg. The LD_{50} of barium, based on Dieke's observations would be about 1030 mg/kg. According to Dervilleé and Raveleau(3), the range in fatal dose of barium, given in the form of carbonate, is 35-56 mg, 104-139 mg, and 418-557 mg, respectively, for the cat, guinea pig, and rabbit, per kilo of live weight. In view of wide differences among tolerances of different mammalian species for barium, it was of interest to study the tolerance of the chicken. Barium hydroxide and barium acetate were chosen for this study because they are among the most soluble of all barium compounds.

Procedure. Two types of experiments, single-dose toxicity and growth, were used. Single, graded doses of barium as barium hydroxide were administered to 40 male chickens 7 weeks old with average weight of 943 $\pm$ 44 g. The required quantities of barium hydroxide to supply 400, 500, 600, 700, and 800 mg of barium were weighed to nearest mg and packed into gelatin capsules. A capsule was placed as far down each chicken's esophagus as possible; then, by stroking the chicken's neck, the capsule was moved into the crop. Procedures in the two growth experiments were similar to those described by Mehring, *et al.*(4). In Exp. 1, 12 lots of chicks (10 males and 10 females per lot) were

TABLE I. Toxicity of a Single Dose of Barium Hydroxide.

Barium (mg)	Survivors/ No. treated	Barium (mg)	Survivors/ No. treated
400	7/8	700	2/8
500	5/8	800	1/8
600	4/8		

given the basal diet (Table II) for the first week. When the chicks were 1 week old, they were weighed, wing banded, and randomly assigned to their respective experimental diets. Levels of barium included in the diet in Exp. 1 ranged from 0 to 1,280 ppm (Table III). In Exp. 2, 16 lots of chicks (20 females per lot) received the experimental diets from one day of age. Both growth experiments were terminated when the chicks were 4 weeks old. Levels of barium and the form in which this element was added to the basal diet in Exp. 2 are shown in Table IV.

Results. Results of single-dose toxicity experiment are shown in Table I. The LD_{50} dose of barium was estimated by method of Gullina, *et al.*(5) to be 587 ± 147 mg/chicken or 623 ± 156 mg/kg live weight.

Results of Exp. 1 and 2 indicate that chickens can tolerate 1,000 ppm of barium in their diet. In Exp. 1 (Table III) average gain in live weight of chickens in all 12 lots, from 1 week to 4 weeks of age, was 321 ± 8 g. Although growth of chickens that received 1,280 ppm of barium in their diet appeared depressed, their average gain (312 g) was approximately only one standard error below average gain of all 12 lots. Accordingly, since no diets contained more than 1,280 ppm of barium, one must conclude that this slight decrease in average gain may have been due to chance.

In Exp. 2 (Table IV) with much higher levels of barium fed, 1,000 ppm of barium in the diet was well tolerated by the chicken, but 2,000 ppm produced slight depression in growth. The 4,000 ppm of barium caused significant depression of growth but did not increase mortality. More than one-half the chickens receiving 8,000 ppm of barium in their diet died before they were 4 weeks old. Chickens that received 16,000 ppm of barium, as barium hydroxide, lived an average of 7 days; and chickens receiving same quantity of barium, as barium acetate, lived an average of 6.8 days. Chickens fed 32,000 ppm of barium as barium hydroxide lived an average of only 5 days.

Gains in live weight of chickens in series receiving barium hydroxide appeared adversely affected with each increase in level of barium hydroxide. However, chickens fed the basal diet in the barium hydroxide series gained considerably more than chickens fed

TABLE II. Formula of the Basal Diet.

Ingredient	%
Ground yellow corn	61.56
Soybean oil meal (44% protein)	31.0
Fish meal (Menhaden)	3.0
Dicalcium phosphate	1.9
Calcite flour	1.7
Salt	.45
25% dry mixture choline chloride	.12
Trace mineral pre-mix (Delamix*)	.10
Vit. B_{12} and antibiotic supplement (Pro-Pen "2:3"†)	.10
Vit. A supplement (Nopcay "10"‡)	.04
" E " (Myvamix§)	.03
" D " ‖	.01
Niacin	.003
Menadione sodium bisulfite	.0005
Calcium pantothenate	.0005
Riboflavin	.0004

* Contained 6% manganese, 0.12% iodine, 2% iron, 0.2% copper, 0.02% cobalt, and 0.01% zinc.
† Contained 2 g of procaine penicillin and 3 mg vit. B_{12}/lb.
‡ Contained 10,000 I.U. vit. A/g.
§ " 20,000 " " E/lb.
‖ " 3,000 I.C.U. vit. D_3/g.

TABLE III. Effect of Barium Hydroxide on Growth and Feed Efficiency of Chickens between 1 and 4 Weeks of Age: Exp. 1.

Level of added barium, ppm	Gain in live wt, g	Gain / Feed
0	329	.510
1.25	305	.494
2.5	318	.510
5	328*	.512
10	320	.498
20	330	.511
40	328	.502
80	319	.477
160	315	.505
320	321	.516
640	331	.512
1280	312	.497

* Only one of 240 chicks died; it was in the lot receiving 5 ppm barium.

TABLE IV. Effect of Barium Hydroxide and Barium Acetate on Growth, Mortality, and Feed Efficiency of Chickens between 1 Day and 4 Weeks of Age: Exp. 2.

	Barium hydroxide series			Barium acetate series		
Level of added barium, ppm	Gain in live wt, g	Mortality	Gain / Feed	Gain in live wt, g	Mortality	Gain / Feed
0	483	1	.554	435	2	.541
250	No chicks at this level			436	2	.566
500	450	0	.538	410	0	.538
1000	428	0	.552	448	0	.535
2000	406	0	.518	428	1	.540
4000	410	1	.537	357	1	.518
8000	231	14	.510	218	12	.485
16000		20			20	
32000		20		No chicks at this level		

the same basal diet in the barium acetate series. Chickens in lots fed diets containing barium hydroxide gained an average of 401 g, while those in lots fed diets containing barium acetate gained 390 g. Thus it appears that the 2 compounds had similar effects on growth of chickens. If the 2 series of lots in Exp. 2 are considered as duplicates, average gain in weight, for same level of barium, indicates that 1,000 ppm of barium was tolerated and that 2,000 ppm produced slight growth depression.

Addition of barium to the diet of chickens had little or no effect on efficiency of feed utilization, at least at levels to 4,000 ppm and possibly to level of 8,000 ppm.

Summary. The LD_{50} dose of barium for young growing chickens was 623 ± 156 mg/kg of live weight. When barium was fed continuously in the feed, either as barium hydroxide or barium acetate, chickens tolerated levels to 1,000 ppm without apparent ill effects. A slight depression in growth occurred when 2,000 ppm of barium was added to the diet. 8,000 ppm of barium caused death of more than one-half of the chicks in 4 week feeding experiment; and the higher levels (16,000 and 32,000 ppm) caused death of all chicks.

1. Hüter, F., *Naturforsch. Z.*, 1946, v1, 418. (*Chem. Abst.*, 1947, v41, 6366c)

2. Dieke, Sally H., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 593.

3. Dervilleé, P., Raveleau, R., *Folia Med.*, 1951, v34, 225. (*Chem. Abst.*, 1952, v46, 2693a).

4. Mehring, A. L., Jr., Brumbaugh, J. H., Sutherland, A. J., Titus, H. W., *Poultry Sci.*, 1960, v39.

5. Gullina, P., Winitz, M., Birnbaum, S. M., Cornfield, J., Otey, M. C., Greenstein, J. P., *Arch. Biochem.*, 1956, v64, 319.

Received May 18, 1960. P.S.E.B.M., 1960, v104.

Relation Between Plasma Iron Turnover and Plasma Iron Concentration at Different Levels of Erythropoiesis. (25867)

G. HODGSON,* D. YUDILEVICH, I. ESKUCHE AND M. PERRETTA
(Introduced by A. S. Gordon)
Seccion Biologica - Laboratorio de Fisica Nuclear, Instituto de Fisica y Matematicas, Universidad de Chile.

When tracer doses of siderophilin-bound Fe^{59} are injected intravenously in rabbits, plasma radioactivity declines as a simple exponential function of time ($Fe^{59} = 1.e^{-Kt}$) (1). The value of time constant K is an estimate of fraction of plasma iron turned over per unit time. Plasma iron turnover is calculated as the product of K, plasma iron concentration and plasma volume. From results of

* Now at California Inst. of Technol., Pasadena.

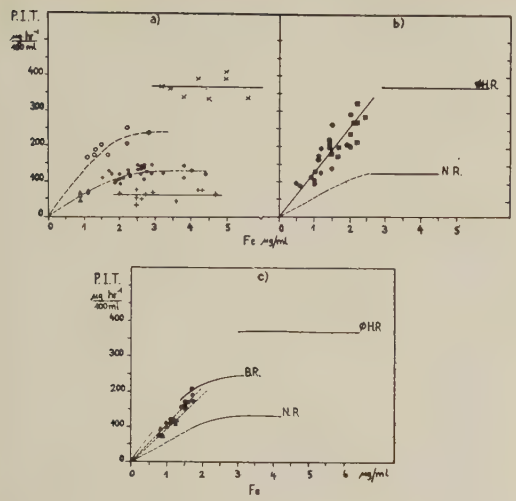


FIG. 1. Relation between plasma iron turnover (P.I.T.) expressed as $\frac{\mu g/hr}{100 \text{ ml plasma}}$, and plasma iron concentration, [Fe].

1 a.) ● Normal rabbit (N.R.). × Rabbit after 5 inj. of phenylhydrazine (ØH.R.). ○ Rabbit (B.R.) 72 hr after 20 ml/kg hemorrhage. △ Rabbit inj. with acetone extract of plasma from bled rabbits. + Fasted rabbits inj. with 0.9% NaCl.

1 b.) ● Rabbit inj. with plasma from phenylhydrazine treated donors. ■ Rabbit inj. with boiled plasma of phenylhydrazine treated donors. N.R., normal rabbits; Ø H.R., phenylhydrazine treated rabbits; B.R., bled rabbits.

1 c.) ▲ Rabbit inj. with plasma of bled donors. ● Rabbit inj. with dialyzed plasma of bled donors.

studies of plasma iron turnover in humans (2), it has been postulated that plasma iron turnover is independent of plasma iron concentration [Fe], and thus that the value of K varies reciprocally with [Fe], and $T\frac{1}{2}\ Fe^{59} = \frac{0.693}{K}$ increases as a linear function of [Fe]. This type of relationship has also been assumed to apply in rabbits(3). This communication presents studies of the relation between plasma iron turnover and plasma iron concentration in rabbits in which level of erythropoiesis was varied by fasting, bleeding, phenylhydrazine injections or treatment with plasma or extracts of plasma from animals rendered anemic by bleeding or phenylhydrazine.

Methods. Plasma iron turnover and Fe^{59} incorporation into erythrocytes were studied in rabbits of both sexes weighing 2-2.5 kg using procedures described previously(1).

Results. Plasma iron turnover and plasma iron concentration. In animals with normal erythropoiesis, which includes untreated controls as well as rabbits which received 0.9% NaCl solution or normal rabbit plasma, P.I.T. is independent of [Fe] over a certain range, but then drops in relation to [Fe] (Fig. 1a). In animals treated with acetone extracts of plasma of bled rabbits, plasma iron concentration is low and so is P.I.T. The experimental points for these animals are close to a line extrapolating the trend in normal animals. These animals had 70% of Fe^{59} in red cells at 48 hours. In fasted rabbits with low P.I.T. and high iron levels, and in phenylhydrazine treated animals with high turnover and high plasma [Fe], P.I.T. is independent of plasma iron concentration. However, in rabbits which had been bled 72 hours previously, P.I.T. increases as function of plasma [Fe]. Both bled and phenylhydrazine treated animals had 90 to 100% Fe^{59} in erythrocytes 48 hours after tracer injection.

P.I.T. and plasma [Fe] in animals treated with plasma or plasma extracts obtained from bled or phenylhydrazinized animals. Fig. 1b shows the relation between P.I.T. and plasma [Fe] in animals receiving plasma or extracts of boiled plasma obtained from phenylhydrazine-treated rabbits. The dependence of P.I.T. on plasma iron is clear. All animals shown had 80-90% Fe^{59} in red cells at 48 hours. In normal rabbits which had received native and dialyzed plasma from severely bled donors (Fig. 1c), P.I.T. is a linear function of plasma iron concentration, and in animals injected with dialyzed plasma from bled donors, P.I.T. and [Fe] are higher than in those animals receiving native plasma.

Discussion. In rabbits with increased erythropoiesis, the ratio between iron going to the marrow and P.I.T. is approximately one. Experimentally the values determined range between 80-100% of Fe^{59} in red cells at 48 hours. Under these conditions plasma iron turnover can be assumed to reflect closely iron uptake by the marrow. Plasma iron turnover is a linear function of plasma iron concentration below about 2 μg/ml (Fig. 1).

These results could be explained by assuming that the limiting factor at low plasma iron concentration is the iron supplied to the marrow by the blood. Uptake of iron (Q_{Fe_M}) by the marrow can be calculated as the product of blood flow to marrow (Q_{b_M}) and the arterial-venous difference in iron concentration ($Q_{Fe_M} = Q_{b_M}[Fe_a - Fe_v]$). When arterial plasma iron level drops below a certain concentration, (Fe_v) might drop to zero; under these conditions ($Q_{Fe_M} = Q_{b_M}[Fe_a]$) marrow iron consumption would be a linear function of Q_{b_M} and plasma iron clearance would be a measure of the blood flow to marrow. The observed clearance values in our experiments suggest that it is highly improbable that blood flow is the limiting factor for, if this were the case, it would mean that only about 2%† of blood volume per minute goes to marrow, a very low figure. Therefore, an explanation for the findings in Fig. 1 may be sought by examining the relation between iron uptake by erythrocyte precursors and iron concentration. It can be postulated that P.I.T. at low plasma [Fe] concentration is a function of plasma iron concentration and number of erythrocyte precursors, including circulating reticulocytes, forming Hb; while at high [Fe] level, P.I.T. is a function only of number of precursors. These postulates are analogous to those used for describing the relation of substrate concentration to rate of an enzymatically catalyzed reaction. In the case of P.I.T., the number of rbc precursors would represent total enzyme. Much experimental evidence points to the existence of an enzymic mechanism incorporating iron into heme(4,5). Recent experiments carried out with reticulocytes(6) and rbc(7) precursors show that iron uptake in reticulocytes is independent of Fe concentration above, and dependent below certain levels(6). The same applies to marrow cultures where at high plasma iron concentration, Fe uptake depends only on number of rbc precursors present while below a certain level it depends linearly on [Fe] for a given number of precursors(7). The hypothesis describing the relation between P.I.T. and plasma [Fe], enables one to infer the erythropoietic activity of marrow under conditions of low or optimal concentration of plasma iron. When plasma iron is optimal, P.I.T. is proportional to number of red cell precursors (P.I.T. $= k_1 \times Np$); when plasma iron is low, P.I.T. is proportional to the product of [Fe] and number of red cell precursors, and plasma iron clearance is a function of the red cell precursors $\left(\frac{\text{P.I.T.}}{[\text{Fe}]} = k_2 \times NP\right)$.

The finding described previously(1) that plasma of severely bled animals does not significantly increase average P.I.T. in normal animals but that a significant increase is seen after injecting dialyzed plasma can be explained, in the light of the present findings, as a consequence of the existence in plasma of severely bled rabbits of a factor, probably dialyzable and extractable in acetone which lowers plasma iron concentration. The fact that plasma Fe^{59} clearance is high in these animals can be taken as evidence of increased number of red cell precursors in the marrow, and thus of enhanced erythropoiesis. The finding of increased reticulocytes in the blood of animals treated with bled rabbit's plasma supports this view(8,9).

Analysis of the different aspects of iron metabolism in bled, and phenylhydrazinized rabbits and in animals injected with plasma extracts with erythropoietic activity, suggests an explanation for the findings that plasma iron is usually low in animals injected with extracts and in bled animals, whereas in animals with hemolytic anemia, it remains normal or high. Plasma iron level is a function of the balance between input and output of iron. The most important input of Fe comes from destruction of red cells, while the most important output is that of iron going to the bone marrow. In bled animals and in those injected with erythropoietically active extracts, iron output increases with no increase of input from destroyed red cells, and plasma iron initially will drop, as output outbalances input, until a level is reached when input from depots and intestinal tract again equals out-

† This value is obtained by taking a value of K of 120%/hr or 2% per minute.

put. In animals with abnormal hemolysis, on the other hand, input from destroyed cells is high and initially above output; plasma iron thus rises to a certain level when, mainly as a consequence of increased red cell production, output again equals input.

Summary. 1) The relation between plasma iron turnover and plasma iron concentration is studied in normal and fasted rabbits, in bled and phenylhydrazine treated rabbits, and in rabbits treated with extracts of plasma of bled and phenylhydrazine-treated donors. 2) In normal, phenylhydrazine-treated and starved rabbits, plasma iron turnover is independent of iron concentration over the range of 2-5 μg Fe/ml; below this range plasma iron turnover in normal rabbits tends to drop. Bled rabbits and rabbits receiving extracts with erythropoietic activity show lower plasma iron concentrations than normal, and in these animals plasma iron turnover depends on iron concentration.

1. Hodgson, G., Eskuche, I., Yudilevich, D., Hernandez, F., Tohá, J., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 826.

2. Bothwell, T. H., Hurtado, A., Donohue, D. M., Finch, C., *Blood,* 1957, v12, 409.

3. Hodgson, G., Yudilevich, D., Perretta, M., Eskuche, I., Tohá, J., *Ann. N. Y. Acad. Sci.,* 1959, v77, 703.

4. Nishida, G., Labbe, R. F., *Biochim. Biophys. Acta,* 1959, v31, 519.

5. Labbe, R. F., *ibid.,* 589.

6. Jandl, J. H., Inman, I. K., Simmons, R. L., Wallen, A., *J. Clin. Invest.,* 1959, v38, 161.

7. Thorup, O., Strcle, W., Leavell, B., *J. Lab. Clin. Med.,* 1958, v52, 266.

8. Hodgson, G., Tohá, J., *Blood,* 1954, v9, 299.

9. Erslev, A., *ibid.,* 1953, v8, 349.

Received December 15, 1959. P.S.E.B.M., 1960, v104.

Effects of Urinary Hemopoietine on Fe^{59} Distribution in Rats Studied While Plasma Fe^{59} is High. (25868)

G. HODGSON,* I. ESKUCHE, S. FISCHER AND M. PERRETTA
(Introduced by A. S. Gordon)
Lab. de Fisica Nuclear, Inst. de Fisica y Matemáticas, and Lab. de Fisiopatologia, Escuela de Med., Universidad de Chile.

In the majority of studies in which Fe^{59} has been used in rats to assay extracts with erythropoietic activity, the criterion employed has been the fraction of injected Fe^{59} (% Fe^{59}) appearing in erythrocytes from 16-24 hours after tracer injection(1,2,3). Although extracts rich in erythropoietic activity increase erythrocyte Fe^{59} uptake, this method does not permit quantification of increase in erythropoiesis. Determination of plasma iron turnover and uptake of iron by marrow would give data more directly related to magnitude of erythropoiesis(4,5). Since, when large numbers of rats are employed, it is not practical to carry out accurate plasma iron turnover measurements requiring serial blood samples, we devised a technic in which plasma iron turnover and mean plasma Fe^{59} specific activity (between 0 - t hours) can be estimated from one plasma sample taken at a time (t hours), when plasma Fe^{59} is still high in controls. From mean plasma specific activity and % uptake by different organs, quantity of iron going to these organs can be calculated. We here describe the technic and its application to assay of urinary erythropoietic activity. The results of this method are compared with 24 hour Fe^{59} assay.

Methods. Extracts with erythropoietic activity were obtained from urine of phenylhydrazinize-treated rabbits and patients with aplastic anemia by methods described elsewhere(6). The kaolin adsorption procedure used was similar to that employed by Winkert *et al.*(7). Grey rats, both sexes of a x c strain obtained from Inst. de Biol. Juan Noé,

*Now at Division of Biology, California Inst. of Technol., Pasadena.

Univ. de Chile, were used either in normal condition or fasted 4 days. Weights are given in the Tables. Injections of extracts were made on 2nd and 3rd day of fasting, and Fe^{59} was injected(3) on morning of 4th day. Some animals were bled at 3 hours after tracer injection when starved animals were used, and at 1½ hours when recipients were normal. Preliminary experiments showed that at these times about 40% of radioiron was still in the plasma. The animals, anesthetized with ether, were bled from the aorta, using a heparinized syringe. Plasma was separated by centrifugation, and red cells were washed thrice with cold 0.9% NaCl solution Radioactivity of red cells, liver and both femurs was measured in well type scintillation counter, and activity was compared with standards of comparable volume. Plasma iron concentration was measured by method of Schade (8). On morning of 5th day of fasting, 24 hours after tracer injection, the rest of the animals were bled and radioactivity in washed red cells was measured. For calculation of percentage injected Fe^{59} in plasma, plasma volume was taken as 3 ml/100 g weight. For calculation of total red cell Fe^{59} uptake, red cell volume was taken as 2.3 ml/100 g weight. Values were obtained from experiments in which red cell volume was measured using erythrocytes tagged with Fe^{59}. Radioactivity in organs was corrected for that contributed by residual blood by factors obtained in animals killed 3 and 6 minutes after injection of serum and red cells tagged with Fe^{59} respectively.

To calculate plasma iron turnover and plasma mean specific activity, it was assumed that in rats(9,10), as in rabbits(5) and humans(4), plasma Fe^{59} radioactivity declines as a simple exponential function of time. Thus: Plasma $Fe^{59} = 1.e^{-Kt}$, where K = plasma iron turnover rate(9), or fraction of plasma iron turned over per unit time. Given plasma Fe^{59} at time t, it is possible to obtain the value of K using tables of exponential function or from $K = 1/3 \ln \frac{1}{F_t}$ (F_t = fraction of injected Fe^{59} remaining in plasma at time t). With the value of K, plasma iron turnover (P.I.T.) $\mu g/$ hr is calculated as: P.I.T. = K x Fe x P_v (Fe = plasma iron concentration, P_v = plasma volume). With these data, mean plasma specific activity $\overline{SA}_p$, between 0-t hours, can be calculated from $\overline{SA}_p = \frac{1}{Fe \times P_v \times t}$ $\int_0^t e^{-Kt}\, dt = \frac{1 - F_t}{P.I.T. \times t}$. From $\overline{SA}_p$ an estimate can be made of amount of iron which flows to any organ from the plasma compartment. Thus iron flux to the liver Q_L equals: $Q_L = \frac{\%\ Fe^{59}L}{\overline{SA}_p \times t}$ (% $Fe^{59}L$ = % injected Fe^{59} in liver at time t). This calculation is based on the assumption that loss of radioiron from liver between 0-3 hrs is negligible. In the case of marrow it is not possible to make this assumption because appreciable amounts of Fe^{59} appear in red cells at 1½ and 3 hours in animals treated with erythropoietically active extracts. In these experiments, the flux of Fe to the marrow Q_m is taken as approximately equal to total amount of iron leaving plasma minus that going to non Hb-forming tissues. The latter was assumed to equal twice the liver iron flux because preliminary studies of Fe^{99} distribution in viscera of the rat, made 3 hrs after tracer injection, demonstrated that liver contained as much radioiron as the sum of that found in heart, lungs, gastro-intestinal tract and kidneys, after appropriate corrections were made for radioactivity contributed by remaining blood. Therefore, Q_m = P.I.T. – 2 Q_L. The validity of this calculation is supported by the finding that in normal rats the fraction of injected Fe^{59} in erythrocytes at 48 hours closely approximates the ratio of Q_m/P.I.T.

Results. (Table I.) In fasted rats injected with urinary hemopoietine plasma radioactivity is much lower than in control rats injected with saline or with extracts of urine from normal rabbits. Estimated values for P.I.T. and Q_m are higher and are a function of amount of extract injected. Erythrocyte Fe^{59} is higher in hemopoietine-treated rats than in saline controls. Animals receiving extracts of urine from normal rabbits have higher erythrocyte Fe^{59} than saline controls and yet have similar P.I.T. and Q_L. Injection of hemopoietine

TABLE I. Fe^{59} Distribution at 3 Hours in Starved Female Rats, and at 1½ Hr Post Tracer Injection in Non-fasted Rats. Values indicated are means and standard errors. Plasma iron concentration (Fe) was measured in samples of pooled plasma. Duplicate measurements agreed within 3%.

	Group	No.	Plasma Fe^{59}	Erythrocyte Fe^{59}	Liver Fe^{59}	Femur, Fe^{59}	E.P.I.T.	E.Q.L.	E.Q.M.	Plasma (Fe)
			% dose					μg/hr		μg/ml
(a)	Fasted ♀ receptors (140 g)	22	27.6 ± 1.0	1.60 ± .17	15.3 ±1.1		6.8	1.45	3.9	3.8
	N.R.U., 6 mg	11	21.0 ± 3.1	4.0 ± .48	18.8 ± .6		6.4	1.29	3.8	2.5
	Hp, 6 mg	8	5.6 ± .50	3.9 ± .58	7.4 ± .42		10.7	.83	9.0	2.7
	,, 12 ,,	6	3.8 ± .38	7.0 ± .30	6.2 ± .51		15.0	.97	13.0	3.3
(b)	Fasted ♀ receptors (180 g)	5	41.2 ± 1.45	.53 ± .11	11.5 ±6	2.7 ±.34	4.8	.9	3.0	3.1
	3 hr post tracer inj. $CoCl_2$, 10 μM	4	32.1 ± 2.13	3.3 ± .95	13.9 ± .72	4.9 ±.43	6.4	1.3	3.8	3.3
	,, 20 ,,	5	16.4 ± 2.67	6.2 ±1.46	13.0 ± .47	6.0 ±.38	7.3	1.2	4.9	2.3
	H.U.Hp	6	3.7 ± 23	30.2 ±1.12	4.2 ± .1	5.4 ±.2	9.4	.4	8.6	1.7
(c)	Non-fasted ♀ receptors (180 g)	7	38.1 ± 1.2	4.5 ± .3	7.3 ± .46	3.6 ±.19	7.8	1.9	4.0	2.3
	1½ hr post tracer inj. $CoCl_2$, 10 μM	6	40.2 ± 1.51	3.5 ± .37	8.9 ± .26	3.2 ±.11	10.1	3.0	4.1	3.3
	,, 20 ,,	6	37.3 ± 1.3	4.4 ± .69	8.6 ± .14	3.3 ±.13	10.8	3.0	4.7	3.3
	Hp (18 mg) Kaolin	7	22.5 ± 1.13	15.2 ±1.49	9.2 ± .58	4.4 ±.36	16.8	3.9	9.0	3.3

E.P.I.T. = Estimated plasma iron turnover. E.Q.L. = Estimated flow of iron to liver. E.Q.M. = Estimated flow of iron to marrow. N.R.U. = Material precipitating with 4 vol ethanol at pH 4.5 from normal rabbit urine. Hp. = Material precipitating at pH 4.5 with 4 vol ethanol from urine of phenylhydrazinize-treated rabbits. H.U.Hp. = Human urinary hemopoietine; material precipitating with 4 vol ethanol at pH 4.5 from urine of patient with aplastic anemia, dose/rat = material obtained from 30 ml urine. HP Kaolin = Hemopoietine obtained from urine of pheuylhydrazine-treated rabbits by adsorption on kaolin, elution with 1 M NH_4OH and precipitation with 5 vol acetone at pH 4.5.

in starved rats increases Femoral Fe^{59} but lowers Liver Fe^{59}. The response of normal rats to hemopoietine (Table Ic) is similar to that of starved rats except for Liver Fe^{59} and Q_L which increases. Table I also shows the effects of 10 and 20 μM $CoCl_2$, a substance known to stimulate erythropoiesis, on Fe^{59} distribution in fasted and normal rats. Like hemopoietine, cobalt decreases plasma Fe^{59} and increases erythrocyte Fe^{59}. However, cobalt differs from hemopoietine in that it increases, rather than lowers Liver Fe^{59} and Q_L in fasted rats. In non-fasted rats the doses of cobalt used do not affect plasma, Erythrocyte Fe^{59} or Femoral Fe^{59}. They do however increase Liver Fe^{59}. Table Ib shows that fasted rats injected with urinary hemopoietine or 20 μM cobalt, which show the most marked changes in Fe^{59} distribution, also have lower plasma iron concentration.

Fe^{59} in red cells is an exponential function of the fraction of plasma iron turned over per hour (%/hr) (Fig. 1a). A 2-fold increase of plasma iron turnover rate is accompanied by an 8-fold increase in Erythrocyte Fe^{59}, while for a 3-fold increase in turnover rate, Erythrocyte Fe^{59} is about 30 times the average control values.

Fig. 1b summarizes results of experiments in which Fe^{59} distribution was studied in 16 groups of fasted male rats (at least 4 animals per group) at 3 hours and in another 16 groups at 24 hours after tracer injection.

FIG. 1a. Relation between erythrocyte Fe^{59} 3 hr post tracer inj. (ordinate), and fraction of plasma iron turned over/hr (K %/hr) (abscissa) in: ■ Fasted controls rats; × fasted rats inj. with preparations of urinary hemopoietine.

FIG. 1b. Relation between % Fe^{59} in erythrocytes at 24 hr (ordinate) and that present at 3 hr (abscissa).

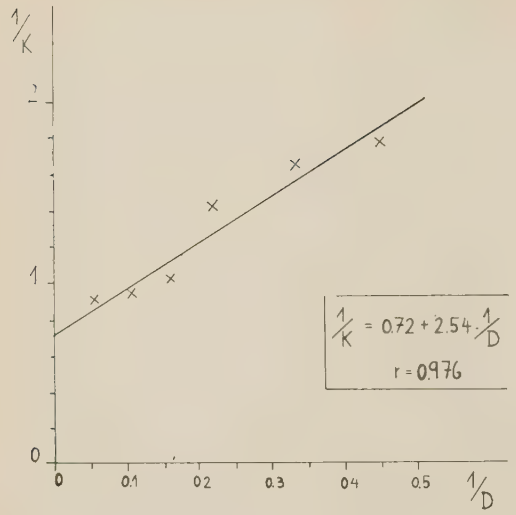


FIG. 2. Relation between reciprocal of K, fraction of plasma iron turned over/hr (ordinate), and reciprocal of dose (D) of rabbit urinary hemopoietine inj. (abscissa).

Seven of the groups were controls, and the other 11 received preparations of urinary hemopoietine from phenyl-hydrazine treated rabbits or patients with aplastic anemia. A significant linear correlation exists between Erythrocyte Fe^{59} at 24 hours and that at 3 hours ($r = 0.985$).

Fig. 2 shows the relation between reciprocal of dose (D) of rabbit urinary hemopoietine and reciprocal of effect as measured by K%/hr, fraction of plasma iron turned over per hour. This type of dose effect analysis was proposed by Stetten(11). The results show a significant linear relationship ($r = 0.976$) between 1/K and 1/D. From the parameters of this line, one may calculate the maximum effect, and dose of rabbit urinary hemopoietine producing ½ maximal effect: 139%/hr and 3.52 mg respectively.

Discussion. The increase in P.I.T. in hemopoietine treated rats probably results from increased flow of iron to bone marrow, evidenced by increased Fe^{59} uptake in femurs and erythrocytes, and decreased flow of iron to the liver.

Hemopoietine injections produce a much larger increase in 3 hr erythrocyte Fe^{59} than in plasma iron turnover rate; in fact the former is an exponential function of the latter. This can be explained by assuming that extracts containing hemopoietine not only increase the plasma iron turnover rate—presumably as a consequence of increased consumption of iron by the marrow—but also shorten mean time spent by reticulocytes in the marrow, as in bleeding and hemolytic anemias(12). The marked effect of hemopoietine on 3 hr erythrocyte Fe^{59} is especially clear in the data in Table Ib, where increase in erythrocyte Fe^{59} is about 60-fold, whereas increase in marrow iron consumption is only about 3-fold. Since 30% of injected radioiron was already in red cells by 3 hours, an appreciable flow of iron went to red cells already in circulation or entering it within the 3 hour period.

The significant linear correlation between 24 hour erythrocyte Fe^{59} and 3 hour Fe^{59} shows that study of Fe^{59} distribution at 3 hours gives as good information on erythrocyte Fe^{59} as 24 hr values. It also permits calculation of plasma iron turnover rate and estimation of marrow iron consumption. The latter data indicate how much Hb synthesis has increased, whereas erythrocyte Fe^{59} values alone do not reflect proportionately the changes in marrow iron consumption, presumably because they are influenced by the time spent by the reticulocyte in the marrow. Moreover, this time may vary independently of consumption of iron by Hb synthesizing tissue, and consequently small increases in erythrocyte Fe^{59} 18-24 hrs after tracer injection may not indicate increase in iron uptake by erythroid tissue.

Increases in plasma iron turnover and marrow iron consumption produced by urinary hemopoietine are influenced by plasma iron concentration at the time of Fe^{59} injection (13). In general we have observed that the more potent the preparation of hemopoietine used, the greater is the decrease in plasma iron concentration (Table Ib). This drop of plasma iron could be a reflection of increased marrow consumption, not exactly balanced by input of iron into the plasma from storage or intestinal tract. This accords with the findings that changes in serum iron level, in absence of hemolysis or iron deficiency, reflect changes in magnitude of erythropoiesis(14) and with the correlation between reticulocyte

and marrow response and fall in plasma iron seen in pernicious anemia after treatment with liver extract(15).

The data obtained from quantitative bioassay carried out by the method of Stetten (11) allow a calculation of maximum effect in this type of assay, and also of the dose producing half maximum effects ($D_{½}$ max.). These parameters may prove useful for intercomparison of various fractions obtained in a purification procedure. If an extract showed unchanged maximum effect and decreased $D_{½}$ max. after fractionation, the material eliminated probably did not have a deleterious effect on erythropoiesis, and the decrease in $D_{½}$ max. would measure increase of specific activity of the preparation. However, if some material with deleterious effect on erythropoiesis were eliminated by fractionation the maximum effect might increase.

Our results suggest that in assays of hemopoietine, more adequate information on the state of erythropoiesis can be obtained from studies of Fe^{59} distribution in plasma, liver, marrow and erythrocytes at a time when plasma radioactivity is high, than from determinations of Fe^{59} in erythrocytes 24 hours after tracer injection. The data obtained by measurement of tracer distribution when plasma radioactivity is high allow an estimate of plasma iron turnover and flow of iron to the marrow to be made, and also indicate changes in mean marrow-to-Hb transit time. Such information is useful in assays using normal rate as receptors, for in these the fraction of Fe^{59} in red cells at 24 hours is already high in the controls, and is not very sensitive to changes in plasma iron turnover, whereas plasma iron turnover is a good index of erythropoiesis(16) and can be estimated from the early Fe^{59} distribution studies.

Summary. Fe^{59} distribution in plasma, red cells, femurs and liver is measured at a time (1½ hours in nonfasted, 3 hours in fasted rats) when plasma radioactivity is high. Plasma iron concentration is also measured, and used with the plasma Fe^{59} value to obtain an estimate of plasma iron turnover. This technic is used for assay of urinary hemopoietine from phenylhydrazine treated rabbits and patients with aplastic anemia. Hemopoietine preparations significantly decrease plasma Fe^{59} and increase femoral and red cell Fe^{59}. Fe^{59} in red cells at 24 hours is a linear function of that present at 3 hours. Increase in Fe^{59} in red cells is much larger proportionately than increase in plasma iron turnover, and thus is not a good reflection of the state of erythropoiesis. A dose effect study shows that the reciprocal of the effect, as measured by fraction of plasma iron turned over per hour, is a linear function of the reciprocal of dose of urinary hemopoietine injected.

1. Plzak, L., Fried, F. W., Jacobson, L., Bethard, W., *J. Lab. Clin. Med.,* 1955, v46, 671.

2. Fried, W., Plzak, L., Jacobson, L., Goldwasser, E., PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 237.

3. Hodgson, G., Perretta, M., Yudilevich, D., Eskuche, I., *ibid.,* 1958, v199, 137.

4. Huff, R., Hennessy, T., Austin, R., Garcia, J., Roberts, J., Lawrence, J. H., *J. Clin. Invest.,* 1950, v29, 1041.

5. Hodgson, G., Eskuche, I., Yudilevich, D., Hernandez, P., Toha, J., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 826.

6. Hodgson, G., Perretta, M., Fischer, S., Eskuche, I., Araya, G., Dinamarca, M., accepted for publication in *Blood.*

7. Winkert, J., Gordon, A. S., Piliero, S. J., Medici, P. T., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 351.

8. Schade, A. L., Oyama, J., Reinhart, R. W., Miller, J. R., *ibid.,* 1954, v87, 443.

9. Berlin, N. I., Huff, R., Hennessy, T., *J. Biol. Chem.,* 1951, v188, 445.

10. Girvin, S. C., Hampton, J. K., PROC. SOC. EXP. BIOL. AND MED., 1959, v100, 481.

11. Stetten, D. W., *Science,* 1956, v124, 365.

12. Finch, C. H., Hanson, M., Donohue, D., *Am. J. Physiol.,* 1959, v97, 761.

13. Hodgson, G., Yudilevich, D., Eskuche, I., Perretta, M., PROC. SOC. EXP. BIOL. AND MED., 1960, v104, 56.

14. Moore, C. V., Doan, C., Arrowsmith, C. A., *J. Clin. Invest.,* 1937, v16, 613.

15. Houghton, B. C., Doan, C. A., *Am. J. Clin. Path.,* 1941, v11, 145.

16. Finch, C. A., *Ann. N. Y. Acad. Sci.,* 1959, v77, 410.

Received December 15, 1959. P.S.E.B.M., 1960, v104.

Influence of CO_2 on Respiratory Metabolism of Ehrlich Ascites Tumor.*† (25869)

Nis I. Nissen (Introduced by J. Kieler)
Fibiger Lab., Biological Division, Copenhagen, Denmark

Although CO_2 has long been considered an unimportant waste product in metabolism of mammalian cells, its significance has been realized more fully during past few years, as a result of the demonstration of CO_2 fixation in cellular synthesis of fatty acids, purines and dicarboxylic acids. Over a long period, *Kieler* and collaborators examined the influence of CO_2 tension on respiratory metabolism of different normal and malignant cells using Cartesian diver technic. These examinations have included the Yoshida rat ascites tumor(1,2,3), Earle's L-strain mouse fibroblasts(1), and normal and malignant human leukocytes(4). Results of experiments on influence of CO_2 upon respiratory metabolism of Ehrlich ascites tumor cells are hereby submitted. Whereas in earlier investigations CO_2 tended to stimulate respiration, the effect upon Ehrlich ascites tumor cells was mainly inhibitory in this respect.

Material and methods. Hypotetraploid and hyperdiploid Ehrlich ascites tumors carried in St/EH and DBA/II mice were employed. Ascites tumor cells were inoculated intraperitoneally and experimented after 2-8 days growth. Mice were sacrificed by cervical fracture and ascites fluid aspirated under sterile conditions. Hemorrhagic fluid was discarded. Stained smears prepared for differential counting showed that 95-99% of cells were tumor cells. Ascites cells were suspended in Ringer-Locke's solution to concentration of 5-6,000 cells/μl. Washing and centrifugation was avoided as this damaged the cells morphologically and lowered respiration. Final cell suspension never contained more than 5% ascites fluid. Ringer-Locke's solution was buffered with tris (hydroxymethyl) aminomethane at concentration of 30 mM. Bicarbonate concentration was adjusted relative to CO_2 in the gas phase to give pH of 7.4. pH value of Ringer-Locke's solution was checked with pH meter before suspension of cells. pH value of final cell suspension was checked with pH paper before introduction into the diver, and in many cases also at end of experiment. No changes in pH could be demonstrated in this way, unless cell concentration exceeded 8000/μl and glucose concentration 5 mM. NaCl concentration varied according to bicarbonate and substrate content to maintain a constant osmotic concentration. Respiration was measured with Cartesian diver technic developed by *Linderstrøm-Lang*(5) and *Holter*(6). The divers were charged at 37°C under water. Solutions were gassed and equilibrated with the gas mixture to be studied during last 24 hours before experiment. Besides CO_2, the gas mixtures contained N_2 and O_2. Concentration of the latter was in all cases 15%. Gassing was repeated after introduction of each seal in the diver. The seals were: bottom seal (0.5 μl 0.15 M bicarbonate solution), lower neck seal (0.5 μl cell suspension), middle neck seal (0.5 μl bicarbonate solution), upper neck seal (0.5 μl paraffin oil), and finally mouth seal provided with hollow glass stopper. A detailed description of filling and gassing methods has recently been given by *Kieler*(3). Oxygen consumption/cell/hour was calculated on basis of initial 3-4 hour period, the linear part of respiration curve.

Results. Results of the influence of CO_2 tension upon endogenous respiration of Ehrlich ascites tumor cells have been summarized in Tables I and II. Two-day-old cells of the tetraploid line were in general not influenced by changes in CO_2 tension (Table I). Six days after transplantation 1% CO_2 had, how-

* This study supported by grants from Anders Hasselbalch Anti-Leukemia Fund, Danish Nat. Research Fund, King Christian X Memorial Fund, Weimann Memorial Fund, and Leukemia Soc.

† The author is indebted to Dr. Jørgen Kieler for continued interest and support. Mariann Bjerregard is acknowledged for excellent technical assistance.

TABLE I. Influence of CO_2 on Endogenous Respiration of 2-Day-Old Tetraploid Ehrlich Ascites Tumor Cells in 3 Experiments.

Oxygen consumption/cell/hr (μl $\times$ 10^{-6})			
CO_2 % in gas phase			
0	.3	1	5
4.21	4.43	4.32	3.76
4.59		3.70	4.25
4.08	3.94	4.13	4.37

ever, an inhibitory effect upon endogenous respiration of diploid as well as tetraploid line (Table II). Experiments with the diploid line showed that 5% CO_2 did not materially increase this inhibition. This result was a surprise, viewed in connection with our former experiments, where the effect of CO_2 was principally stimulatory to respiration. In these experiments evidence was obtained that the stimulatory effect of CO_2 on respiration of Yoshida cells is caused by increased synthesis of oxalacetate(2,3). An increased oxalacetate synthesis might also be expected to have a moderate stimulatory effect upon respiration of Ehrlich cells, as oxalacetate, added to the medium in 10 mM concentration, had such an effect in a CO_2-free atmosphere (Table III). It is therefore conceivable that lack of stimulatory effect of CO_2 upon respiration of Ehrlich cells is due to lack of pyruvate or phosphoenolpyruvate, substances with which CO_2 can react to form oxalacetate.

To investigate this possibility, CO_2 effect was examined after addition of glucose and pyruvate. Furthermore the relationship between CO_2 effect and oxalacetate and butyrate was studied. Table III shows results of typical experiments. Addition of glucose, pyruvate and oxalacetate did not reduce CO_2 effect significantly, but respiratory stimulation by pyruvate and oxalacetate, usually seen at room air, was abolished in presence of CO_2.

TABLE II. Influence of CO_2 on Endogenous Respiration of 6-Day-Old Ehrlich Ascites Tumor Cells.

	O_2 consumption/cell/hr (μl $\times$ 10^{-6})		
	CO_2 % in gas phase		
	0	1	5
Diploid cells (5 exp.)	2.12	1.47 (31% $\pm$ 6)*	1.37 (35% $\pm$ 14)
Tetraploid cells (6 exp.)	4.04	2.34 (42% $\pm$ 11)	

* Figures in parentheses indicate % inhibition by CO_2 $\pm$ S.D.

As demonstrated by *Medes and Weinhouse* (7) and others, endogenous respiratory metabolism of Ehrlich ascites tumor cells is concerned mainly with oxidation of fatty acid components. It is therefore interesting that addition of butyrate at concentration of 10 mM is capable of reducing inhibitory effect of CO_2 (Table III) although butyrate, as with other fatty acids(8), has an inhibitory effect upon endogenous respiration.

In view of reports of the significance of biotin in CO_2 fixation, it seemed necessary to exclude the possibility of a lack of biotin in Ehrlich cells. However, neither pretreatment of mice with biotin (25 mg/kg orally or intraperitoneally), nor addition of biotin to the cell suspension had any influence upon CO_2 effect.

TABLE III. Influence of CO_2 on Respiration of Tetraploid Ehrlich Ascites Tumor Cells in Presence of Various Substrates.

			CO_2 % in gas phase		
			0	1	
Age of tumor cells (days)	Substrate	Conc. (mM)	Oxygen consumption/ cell/hr (μl $\times$ 10^{-6})		Inhibition (%)
4	Glucose	0	3.02	1.99	34
		1	4.02	3.08	23
		5	2.44	1.79	27
5	Pyruvate	0	3.74	2.07	45
		5	4.96	2.18	56
5	Oxalacetate	0	4.26	2.61	39
		10	5.09	2.24	56
5	Butyrate	0	4.44	2.42	45
		10	3.27	2.92	11

Discussion. Significance of CO_2 tension in respiratory metabolism of animal cells has been little investigated, and the results are partly contradictory. *Root*(9,10,11) examined Arbacia eggs and nervous and muscle tissue and found in all 3 cases an inhibitory effect of CO_2 upon respiration, which agrees with our results. Several other authors found a stimulatory effect of CO_2/HCO_3^- upon respiration. *Danes and Kieler*(1) and *Bicz*(4) reviewed the literature. Our previous examination(1,2,3,4) has shown, for Earle's L-strain mouse fibroblasts, Yoshida ascites tu-

mor cells and normal and malignant leukocytes, a maximal respiration at 0.5-3% CO_2 in the gas phase. Contrary to *Bicz*(4), *Seelich*(12) demonstrated a stimulatory effect upon normal but no effect upon leukemic leukocytes. *Seelich and Letnansky*(13) found endogenous respiration of Ehrlich ascites tumor cells 8% higher in Krebs-Ringer-bicarbonate solution than in Krebs-Ringer phosphate. *Kieler*(3) demonstrated in Yoshida cells a relation between CO_2 effect and metabolic condition of the cells. Cells with a high mitotic coefficient and low respiration showed a great increase in oxygen consumption in presence of CO_2, whereas cells with high respiration and low mitotic coefficient were less stimulated or even inhibited by CO_2.

It is not possible from our experiments to make definite conclusions as to the mechanism of inhibitory effect of CO_2 upon respiration. However, the experiments with butyrate seem to indicate a relationship to fatty acid metabolism. In this connection, *Miller* (14) found a stimulatory effect of bicarbonate upon incorporation of acetate-C^{14} into adipose tissue. *Gibson, Titchener and Wakil*(15) demonstrated in fatty acid synthesis an absolute requirement for bicarbonate, which seemed to play a catalytic role. It is now generally accepted that the initial step in fatty acid synthesis, as pointed out by *Brady*(16), is a fixation of active CO_2 to acetyl CoA with formation of malonyl CoA. In the equilibrium between fatty acid oxidation and fatty acid synthesis, nutritive and hormonal factors are known to play an important role. Whether this equilibrium is influenced by CO_2 remains to be shown.

Summary. The effect of CO_2 on respiratory metabolism of Ehrlich ascites tumor cells was studied with the Cartesian diver technic. In absence of substrate and in presence of glucose, pyruvate or oxalacetate, CO_2 had a pronounced inhibitory effect upon respiration. Only butyrate addition was found to neutralize CO_2 inhibition. The stimulatory effect upon respiration shown for pyruvate, oxalacetate and glucose (at a 1 mM concentration) in a CO_2 free atmosphere, could, when CO_2 was present in the gas phase, be demonstrated only for glucose.

1. Danes, B. S., Kieler, J., *Compt. rend. Lab. Carlsberg,* 1958, v31, 61.
2. Kieler, J., *Acta U.I.C.C.,* 1960.
3. ———, *J. Nat. Cancer Inst.,* 1960, (July number).
4. Bicz, W., *Cancer Res.,* 1960, v20, 184.
5. Linderstrøm-Lang, K., *Compt. rend. Lab. Carlsberg, Ser. Chim.,* 1943, v24, 333.
6. Holter, H., *ibid.,* 1943, v24, 399.
7. Medes, G., Weinhouse, S., *Cancer Res.,* 1958, v18, 352.
8. Volk, M. E., Millington, R. H., Weinhouse, S., *J. Biol. Chem.,* 1952, v195, 493.
9. Root, W. S., *Biol. Bull.,* 1930, v59, 48.
10. ———, *J. Cell. Comp. Physiol.,* 1932, v1, 239.
11. ———, *Physiol. Zool.,* 1933, v6, 137.
12. Seelich, F., Letnansky, K., Frisch, W., Schneck, O., *Z. Krebsforsch.,* 1957, v62, 1.
13. Seelich, F., Letnansky, K., *ibid.,* 1960, v63, 236.
14. Miller, J. P., Cooper, J. A. D., *Biochim. Biophys. Acta,* 1959, v33, 436.
15. Gibson, D. M., Titchener, E. B., Wakil, S. J., *ibid.,* 1958, v30, 376.
16. Brady, R. O., *Proc. Nat. Acad. Sci.,* 1958, v44, 993.

Received February 23, 1960. P.S.E.B.M., 1960, v104.

D-Amino Acids as Source of Non-Specific Nitrogen for Growth of Rats. (25870)

M. RECHCIGL, JR.,* J. K. LOOSLI AND H. H. WILLIAMS
Depts. of Biochemistry and Nutrition and Animal Husbandry, Cornell University, Ithaca, N. Y.

Feeding of essential amino acids alone, each at its required level, will produce only limited growth of rats. Normal growth is obtained by supplementing such regimen with non-es-

* Present address: Lab. of Biochemistry, Nat. Cancer Inst., N.I.H., Bethesda, Md.

TABLE I. Composition of Amino Acid Mixtures.

Amino acid	Mixture-1*L* Form	g	Mixture-1*DL* Form	g	Mixture-2*L* Form	g	Mixture-2*DL* Form	g
Arginine	*L*. HCl	.93	*L*. HCl	.93	*L*. HCl	2.12	*L*. HCl	2.12
Histidine	*L*. HCl	.35	*DL*. HCl	.35	*L*. HCl	.79	*DL*. HCl	.79
Isoleucine	*L*	.46	*DL*	.46	*L*	1.04	*DL*	1.04
Leucine	*L*	.85	*DL*	.85	*L*	1.93	*DL*	1.93
Lysine	*L*. HCl	1.25	*DL*. HCl	1.25	*L*. HCl	2.84	*DL*. HCl	2.84
Methionine	*L*	.22	*DL*	.22	*L*	.50	*DL*	.50
Phenylalanine	*L*	.48	*DL*	.48	*L*	1.09	*DL*	1.09
Threonine	*L*	.51	*DL*	.51	*L*	1.16	*DL*	1.16
Tryptophan	*L*	.10	*DL*	.10	*L*	.23	*DL*	.23
Valine	*L*	.72	*DL*	.72	*L*	1.63	*DL*	1.63
Tyrosine	*L*	.38	*DL*	.38	*L*	.86	*DL*	.86
Cystine	*L*	.20	*L*	.20	*L*	.45	*L*	.45
$NaHCO_3$		.99		.99		2.51		2.51
Total		7.44		7.44		17.15		17.15

sential amino acids. Experiments in our laboratory(1,2) and elsewhere(3) have shown that the requirement for non-essential amino acids is relatively non-specific, since increase in level of essential amino acids or addition of a single or a few non-essential amino acids will also significantly stimulate growth of animals. Moreover, ammonium compounds and other simple nitrogenous substances, including urea, can be effectively used as nitrogen sources for biosynthesis of non-essential amino acids. Our object was to find out whether *D*-amino acids, as supplied in a racemic mixture of essential amino acids, can serve as source of nonspecific nitrogen. Besides its scientific interest, our study has some practical implications in view of the relatively low cost of *DL*-amino acids compared with expensive *L* enantiomorphs.

Methods. Weanling albino rats of Yale and Holtzman strains were used. Each animal was housed in separate cage and allowed to eat and drink *ad lib*. The rats were divided into experimental groups of 4 rats each and totaling same weight at onset of treatments. Body weight changes, food and water consumption of animals were recorded daily during 10-day experimental period. A basal nitrogen-free diet was compared with various amino acid diets prepared by inclusion of appropriate amino acid mixture and by adjusting percentage of dextrin. Composition of basal nitrogen-free diet has been described (4). Amino acid mixtures (Table I) were composed of essential amino acids, including cystine and tyrosine, and were patterned after the composition of the rat carcass(1). All amino acids were present in their natural *L* form in Mixture-1*L* and in the racemic form in Mixture-1*DL*, except for *L*-arginine and *L*-cystine. Mixture-2*L* and Mixture-2*DL* were comparable to above mixtures except that the level of each amino acid was approximately doubled. Mixtures-1*L* and -1*DL* were calculated to contain 0.93 g nitrogen while the latter mixtures contained 2.11 g nitrogen. Amino acids used were commercial products. A mixture of *L* with *D* alloisoleucine was purchased from Nutritional Biochemicals Corp., Cleveland, O. All other amino acids including racemic and *L* and *D* forms of isoleucine were obtained from Mann Research Lab., N.Y.

Results. Comparative growth response of rats fed natural and racemic amino acids is summarized in Table II. Feeding of a mixture containing essential *L*-amino acids each at its minimum level resulted in average weight gain of 12 g. When concentration of all essential amino acids was approximately doubled growth rate of rats was increased to 23 g. Feeding racemic mixtures of amino acids at either level, however, produced growth inhibition, which was accompanied by anorexia and by lowered food and nitrogen efficiency.

Further studies were conducted regarding the nature of growth inhibition. That animals lost slightly less weight on a diet containing a higher level of amino acids pointed

TABLE II. Comparative Growth Response of Rats to Natural and Racemic Amino Acids.

Series No.*	Nature of diet†	10 day wt gain (g)	Intake: Food (g)	Intake: Water (ml)	Intake: N. (g)	Efficiency: Food	Efficiency: N.	Efficiency: NNR‡
I	Nitrogen-free	−13 ± .2§	51	64		−.26		
	1 *L*	12 ± 1.2	75	127	.693	.16	17	36
	2 *L*	23 ± 4.0	71	152	1.498	.32	15	24
	1 *DL*	−11 ± 2.2	30	64	.277	−.38	−41	6
	2 *DL*	− 7 ± 2.5	31	80	.659	−.22	−11	9
II	Nitrogen-free	− 9 ± .7	35	40		−.27		
	2 *DL* (*L*-isoleucine allo free, *L*-leucine, *L*-valine)	19 ± 1.5	54	137	1.134	.35	17	25
III	Nitrogen-free	− 7 ± 1.0	34	61		−.22		
	2 *DL* (*L*-valine)	2 ± 2.0	33	75	.686	.05	3	13
	" (*L*-leucine)	− 1 ± .5	31	77	.654	−.02	−1	11
	" (*L*-isoleucine allo free)	21 ± 4.0	58	147	1.224	.35	17	23
IV	Nitrogen-free	−15 ± 3.2	40	56		−.36		
	2 *DL* (1.04 g *DL*-isoleucine)	0 ± 1.2	44	80	.928	.00		16
	" 2.08 g "	23 ± 3.0	62	162	1.376	.36	16	27
	" 4.16 g "	24 ± 1.2	63	171	1.468	.38	16	26
	" (.46 g *L*-isoleucine allo free)	25 ± .7	72	174	1.466	.34	17	27
	" 1.04 g *Idem*	30 ± 2.7	78	182	1.651	.38	18	27
	" (1.04 g *L* with *D*-allo-isoleucine)	28 ± 3.0	71	183	1.488	.39	19	29
	" (.52 g *L*-isoleucine allo free + .52 g *D*-isoleucine)	22 ± 2.0	68		1.424	.32	15	26
	" (isoleucine-free)	−18 ± 1.2	32		.635	−.55	−28	−5
	" (.96 g *DL*-leucine)	1 ± 1.5	45	90	.897	.02	1	17
	" (.96 g *L*-leucine)	−1 ± 2.2	46	86	.922	−.02	− 1	15
V	2 *DL* (.26 g *L*-isoleucine allo free)	1 ± .7	52	94	1.056	.02	1	
	" (*Idem* + .26 g *D*-isoleucine)	0 ± 3.3	49	91	1.005			
	" (.52 g *L*-isoleucine allo free)	21 ± 2.3		190				
	" (*Idem* + .52 g *D*-isoleucine)	20 ± 1.3		210				

* In series I, II, III, and V rats of Yale strain were used whereas in series IV Holtzman rats were employed.

† Modification of basal amino acid mixture is given in parentheses.

‡ $\frac{\text{Change in wt of test group + loss of wt of nitrogen-free group}}{\text{Nitrogen intake of test group}}$.

§ Stand. error of mean.

to the presence of a possible dietary deficiency rather than to a toxicity.

When leucine, isoleucine and valine in the racemic Mixture-2*DL* were replaced by their natural enantiomorphs, growth inhibition was overcome. Anorexia which accompanied growth inhibition was also reversed. Substitution of *L*-valine or *L*-leucine alone for their respective racemic forms did not affect growth of rats. Complete reversal of growth retardation was obtained, however, by substituting the *L* isomer of isoleucine for *DL*-isoleucine. Rats ingesting a diet containing this modified racemic mixture gained 21 g of weight which is comparable to weight gain of 23 g on a diet containing all amino acids in the *L* form.

Moreover food and nitrogen efficiency values included in Table II show that these mixtures are equally efficient.

Experiments were also designed concerning nature and possible causes of apparent isoleucine deficiency. Isoleucine has 4 possible optical isomeric forms. Those which were available to us were tested for their effect on growth of rats. These studies showed that growth inhibition could be overcome by replacing *DL*-isoleucine, fed at a level of 1.04 g/100 g diet, by 1.04 g as well as by 0.45 g of allo-free *L*-isoleucine. Feeding of 1.04 g *L* with *D* allo isoleucine produced growth comparable to ingestion of allo-free *L*-isoleucine fed at same level. It was further found that feeding of *D*-isoleucine together with the *L* isomer did not significantly alter growth of rats, and moreover, that growth inhibition on the racemic diet could be prevented by increasing amount of original *DL*-isoleucine.

It may be concluded that the observed isoleucine deficiency resulted from use of an isoleucine preparation which was actually a mixture of 4 isomers of which only L-isoleucine is biologically active(5).

In view of known antagonistic effect of leucine on utilization of other amino acids, especially isoleucine and valine(4,6,7), and that the rat can utilize appreciable amounts of *D*-leucine(4) there was still a possibility that the isoleucine deficiency in our study was aggravated by an excess of leucine in the diet. This was not, however, the case since no beneficial effect resulted when leucine level was decreased or when *L*-leucine was substituted for the racemic form.

Assuming that only 25% of the original *DL*-isoleucine *i.e.* 0.26 g/100 g diet was biologically active, and that presence of other isoleucine isomers does not affect utilization of *L*-isoleucine, one would anticipate that intake of 0.26 g *L*-isoleucine would be insufficient to support growth of rats. The experimental data (series No. 5) support the validity of this conclusion. It is also apparent from the results that *D*-isoleucine does not inhibit utilization of *L*-isoleucine.

Discussion. Since *D*-amino acids are not usually contained in foodstuffs, it has been frequently assumed that feeding of racemic mixtures of amino acids may be harmful. In favor of this view are occasional observations of growth inhibition in experimental animals fed such mixtures(8,9,3). The present study may furnish one possible explanation of how such a growth inhibition may occur.

The experiments confirm the previous finding of Greenstein *et al.*(5) that of 4 known isomers of isoleucine only the *L* enantiomorph, free of allo form, supports growth of rats. *DL*-isoleucine obtained commercially may come as a mixture of either 2 or 4 isomers and presence of the allo forms in such samples is often not stated. Use of such preparations in research may lead to erroneous conclusions.

All available evidence points against the idea that the observed isoleucine deficiency was aggravated by presence of unnatural isoleucine isomers in the diet or by a possibility of amino acid imbalance, which could have arisen from too much leucine in the diet.

The data show that rats are able to utilize *D*-amino acids as a source of nitrogen for biosynthesis of nonessential amino acids. In fact efficiency values indicate that in this respect they are equivalent to their natural *L* antipodes. Prior to this work, Phillips and Berg(10) observed that supplementation with *D* isomers of lysine, threonine, leucine, isoleucine and valine accelerates growth of rats beyond that observed on *L*-amino acids alone.

The observation that *D*-amino acids in the racemic mixture can furnish nitrogen for synthesis of non-essential amino acids is of fundamental importance, since it was customary in the past to disregard the nitrogen supplied by the *D* forms except those which could be easily inverted to the *L* antipodes. This will have a great effect on the ratio of essential to non-essential amino acid nitrogen in many older amino acid diets studied.

Summary. Rats fed a diet containing physiological amounts of essential *L*-amino acids but lacking non-essential amino acids grew at a slow rate. Growth rate was increased by increasing concentration of all essential amino acids. Feeding a mixture of *DL*-amino acids in comparable amounts resulted in growth retardation of animals. Growth in-

hibition produced by the racemic mixture was traced to an isoleucine deficiency resulting from inclusion of *DL*-isoleucine, which was actually a mixture of all 4 isomers, thus effectively diluting the active isomer. Substitution of *L*-isoleucine or a mixture of *L*- and *D* allo-isoleucine for the racemic compound in the *DL* mixture entirely overcame inhibition and produced growth comparable to that obtained with *L*-amino acids. The experiments provide evidence that *D*-amino acids can be effectively used as source of nitrogen for biosynthesis of non-essential amino acids.

1. Rechcigl, M., Jr., Loosli, J. K., Williams, H. H., *J. Nutrition,* 1957, v63, 177.

2. Rechcigl, M., Jr., Kwong, E., Barnes, R. H., Williams, H. H., PROC. SOC. EXP. BIOL. AND MED., 1959, v101, 342.

3. Frost, D. V., in *Protein and Amino Acid Nutrition,* Albanese, A. A., Ed., Academic Press, N. Y. and London, 1959, p225.

4. Rechcigl, M., Jr., Loosli, J. K., Williams, H. H., *J. Biol. Chem.,* 1958, v231, 829.

5. Greenstein, J. P., Levintow, L., Baker, C. G., White, J., *ibid.,* 1951, v188, 647.

6. Wretlind, K. A. J., *Acta physiol. Scand.,* 1956, v36, 119.

7. Harper, A. E., Benton, D. A., Elvehjem, C. A., *Arch. Biochem. Biophys.,* 1955, v57, 1.

8. Van Pilsum, J. F., Berg, C. P., *J. Biol. Chem.,* 1950, v183, 279.

9. Berg, C. P., in *Protein and Amino Acid Nutrition,* Albanese, A. A., Ed., Academic Press, N. Y. and London, 1959, p57.

10. Phillips, W. A., Berg, C. P., *J. Nutrition,* 1954, v53, 481.

Received March 7, 1960. P.S.E.B.M., 1960, v104.

Cholesterol Estimation on Unmeasured Drops of Whole Blood.* (25871)

ANCEL KEYS, MARIO MANCINI† AND JOSEPH T. ANDERSON

Lab of Physiological Hygiene, University of Minnesota School of Public Health, Minneapolis

Risk of future coronary heart disease in middle-aged men is related to concentration of cholesterol in serum measured in apparent health(1,2,3). Accordingly, blood cholesterol measurement is indicated, both in research programs to define relationships more precisely in populations, and in screening and possible preventive programs. Measurement of serum cholesterol is readily made though reports from routine laboratories indicate the need for control(4). But practical requirements limit wide application of cholesterol measurements to scattered populations and insurance applicants. Complications of storing and transporting liquid serum may be avoided by drying a measured amount (0.1 ml) of serum on filter paper, in which cholesterol may accurately be estimated at leisure in another laboratory(5). This requires drawing blood, separating and accurately measuring serum onto paper. These seemingly unexacting requirements are not readily met everywhere and, in fact, impede programs involving cooperation of general practitioners or untrained assistants. To simplify the procedure we studied what may be done with a few drops of finger tip blood, collected on filter paper. The result is a method, adequate for many screening and epidemiological purposes, that makes only trivial demand except at a distant analytical laboratory. Proceeding from the knowledge that drops of serum on filter paper, dried in room air for an hour or 2, may be successfully analyzed for cholesterol months later, trials were made with whole blood; this proved to be stable. The question, then, was to estimate amount of blood used when no measurement is made at time of drawing. Advantage may be taken of the relative constancy in whole blood, except in persons with frank anemia or polycythemia, of concentration of iron, sodium or simply the solids.

Method. Discs of filter paper 4.25 cm di-

* Aided by grants from Minn. Heart Assn. and Am. Heart Assn.

† Present address: Istit. di Patologia Speciale Med. Univ. di Napoli, Italy.

ameter are used (Whatman No. 1 or No. 42 if iron is to be measured). They should be handled with forceps. If whole blood solids are the point of reference, discs are brushed, numbered, dried in vacuum desiccator at least 6 hours, and weighed to 0.1 mg, taking precautions to prevent or correct for moisture absorption after removal from desiccator. We used a micro-torsion balance in dry chamber under slight positive pressure of specially dried nitrogen, manipulation made with rubber gauntlets let into wall of chamber. After weighing, discs are transferred to individual glassine envelopes for mailing or storage. Finger tip is cleaned with alcohol and, when dry, is pricked, allow drops of blood to dribble onto filter paper held with hemostat, then hung by finger loops of hemostat in room air. When paper appears dry (usually 1 to 2 hours) it is returned to glassine envelope and ready for mailing. If either iron or sodium are to be used, weighings are unnecessary. Analysis may be carried out after blood is collected, no refrigeration is required. The paper is removed (forceps) from envelope and again dried and weighed. Cholesterol analysis follows same procedure(5). This involves treatment of paper and blood with alcoholic KOH, extraction with petroleum ether, evaporation of ether extract and development of color with Liebermann-Burchard reagent(6). If iron is to be determined, water and alcohol remaining with residue after petroleum ether extraction are driven off by heat and stream of air. Iron is determined according to method of Snell and Snell(7).

Results. Dried whole blood vs. *serum.* The first series of experiments utilized blood from arm vein from 38 men to provide a wide range of serum cholesterol values. No obvious cases of marked anemia or liver disease were included but the series was not specially screened and included patients with coronary heart disease. Immediately after drawing blood, portions (0.1 ml) were placed on weighed filter paper discs, hematocrit and hemoglobin values were obtained and serum separated for analysis by our version(5) of the method of Abell, *et al.*(6), and also used to measure cholesterol in fresh whole blood. Paper discs were reweighed after drying and, following a few days of storage at ordinary room temperature, were analyzed for cholesterol, as indicated above, and for iron.

TABLE I. Regression Obtained with 38 Blood Samples Covering Ranges 135 to 318 mg Cholesterol/100 ml Serum, Hematocrit Values of 43.6 to 54.1%, and Hemoglobin Values of 13.5 to 17.2 g/100 ml.

	Equation	S.D.
1.	$Hb = 5.81\,Fe + 1.74$	.58
2.	$Vc = 16.1\,Fe + 12.6$	2.04
3.	$W = 21.6\,C + 10.1$	8.00
4.	$Y = 38.9\,C - 84.5$	18.1
5.	$Y = 43.2\,C + 5.04\,Vc - 368$	13.6
6.	$Y = 44.2\,C + 17.3\,Hb - 386$	12.9
7.	$Y = 40.5\,C + 82.8\,Fe - 282$	16.2
8.	$Y = 71.1\left(\frac{C}{Fe}\right) - 24.1$	19.5
9.	$Y_{1,2} = Y_{1,1}$	5 *

* Stand. error of measurement.

Abbreviations: Y = cholesterol, mg/100 ml fresh serum. W = cholesterol, mg/100 ml whole blood. C = cholesterol, mg/mg dried whole blood solid. Fe = iron, mg/mg dried whole blood solid. Hb = hemoglobin, g/100 ml fresh whole blood. Vc = hematocrit, ml/100 ml fresh whole blood. S.D. = stand. dev. from regression, same units as of predicted item (left hand of equation).

Table I summarizes results in terms of regression equations obtained by least squares and standard deviations from regression. Results show that concentration of cholesterol in mg/100 ml of fresh serum can be estimated from cholesterol/unit weight of dried whole blood solid by means of equation No. 4, standard deviation from regression being 18.1 mg/100 ml, or 7.6% of mean of 238.8 mg/100 ml in these 38 samples. As expected, hemoglobin, hematocrit and iron weight/unit weight of dried whole blood are closely related. Since cholesterol concentration/unit of cell volume is remarkably constant(8), inclusion of some measure of cell volume in an estimating equation should improve estimation by correcting for variability in hematocrit. S. D. values associated with multiple regression equations No. 5, 6, and 7 show this, though improvement gained by adding Fe data (with S.D. = 16.2) is not great as compared with prediction omitting this (S.D. = 18.1).

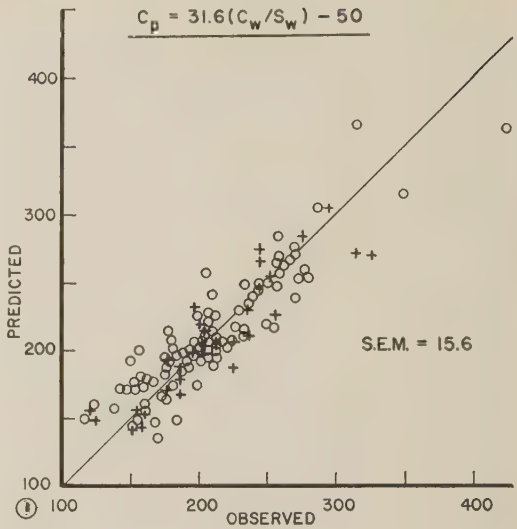
Equation No. 8 is least squares regression

equation for estimation of serum cholesterol from ratio of cholesterol to iron in dried whole blood. The standard deviation of 19.5 mg cholesterol/100 ml of serum for the difference between observed and predicted values is larger than in estimation from cholesterol/unit of whole blood solid (equation No. 4) and suggests that iron as sole reference point is less satisfactory than blood solids.

Results with equations 4 through 8 may be compared with equation 9 which indicates average standard error of measurement when duplicate analyses are made with the standard method of aliquots of the same serum. But, as shown below, the standard error of measurement in equation No. 9 gives a false impression of reliability of single serum cholesterol measurement in estimation of the true mean value of the individual.

Estimation from finger tip blood. The foregoing refers to blood drawn from arm vein by syringe. A new series of 93 men was studied to discover relationship between cholesterol concentration in arm vein blood serum and that per unit weight of whole blood solids dried on filter paper, using first 3 to 5 drops of finger tip blood. As before, men included coronary patients as well as men ostensibly healthy or with only minor complaints; ages ranged from 20 to 65, mean = 53 years. Directly measured serum values ranged from 117 to 423 mg/100 ml, mean = 210.6, and these were obtained independently by different technicians than those who worked with dried whole blood.

The coefficient of correlation between cholesterol concentration directly measured in serum (mg/100 ml) and that measured in dried spots (mg/g of solid) was r = 0.892 in this series and the regression equation was obtained by least squares: 10. $\tilde{Y} = 31.6\ X - 50$ where $\tilde{Y}$ is predicted cholesterol in mg/100 ml of serum and X is that in spots, mg/g of solids. Standard error of measurement, considering observed and predicted values of serum cholesterol as 2 measurements of the same item and computing S.E.M. as usual in that situation ($S.E.M.^2 = \Sigma\Delta^2/2N$), gave S.E.M. = ± 15.6 mg/100 ml.



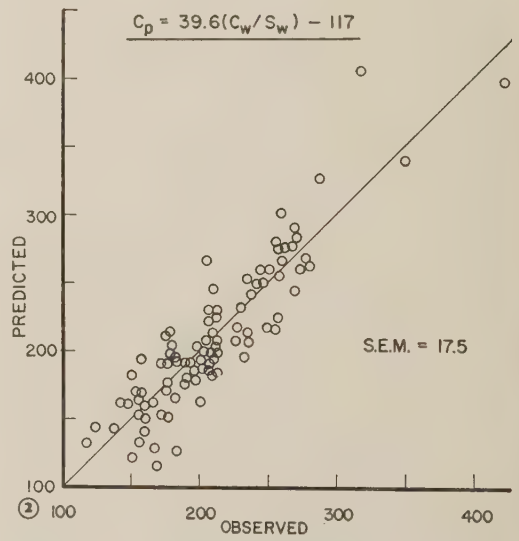


FIG. 1. Observed serum cholesterol measured in arm vein blood plotted against value predicted from ratio of cholesterol to whole blood solids, using the least squares equation (No. 10). ○ = 93 samples used in derivation of equation No. 10. + = subsequent trials with other men.

FIG. 2. Same as Fig. 1 but predicted values were obtained by use of the theoretical equation No. 11a.

Fig. 1 shows serum cholesterol values observed and predicted from equation 10 in this series of 93 samples plus 31 samples analyzed later and not included in derivation of equation No. 10.

Prediction equation from theory. A theoretical equation for prediction of serum cholesterol from cholesterol/unit weight of dried

whole blood can be derived: Let C be concentration of cholesterol in mg/100 ml, with subscripts p, c, and w representing serum (or plasma), cells, and whole blood respectively. Let S be weight of solids in g/100 ml, with subscripts as above. Now, if H is hematocrit, ml of cells/ml of whole blood, we have: $C_w = HC_c + (1–H)C_p$, and $S_w = HS_c + (1–H)S_p$. Accordingly, concentration of cholesterol in mg/g of whole blood solids will be: $\frac{C_w}{S_w} = \frac{HC_c + (1-H)C_p}{HS_c + (1-H)S_p}$, from which concentration of cholesterol in mg/100 ml of serum is, 11. $C_p = \frac{C_w}{S_w}\left(\frac{HS_c}{1-H} + S_p\right) - \frac{HC_c}{1-H}$. This corresponds to regression equation: $C_p = a + b\frac{C_w}{S_w}$, where $a = -(HC_c)/(1–H)$ and $b = (HS_c)/(1–H) + S_p$. We found $C_c = 137$ mg/100 ml with great constancy in human blood(8) so coefficient a may be computed for various values of the hematocrit, H. Computation of coefficient b requires estimates of concentration of solids in serum (S_p) and in cells (S_c). Ranges of these variables to cover 95% of bloods of adults are (9): $S_p = 7.9$ to 9.1, $S_c = 34$ to 39. Mid-points of these ranges, 8.5 and 36.5, respectively, correspond closely to our own findings for medians for adult men aged 20 to 65. Using these values, coefficient b may also be computed for various levels of hematocrit.

In our series of 93 samples to obtain equation No. 10 the hematocrits are not known but mean value may be estimated from equation 11 since we have directly measured mean $C_p = 210.6$ from which H = 0.46 ml cells/1 ml of whole blood and the numerical solution of equation No. 12 is: 11a. $C_p = 39.6\ C_w/S_w - 117$. When equation 11a is applied to the data from the 93 samples the difference between means of observed and estimated serum cholesterol values is 0.26 mg/100 ml, and S.E.M. ± 17.5. Observed and predicted values are shown in Fig. 2. This theoretical estimating equation is almost as good for prediction as the empirical least squares solution, in spite of superficial difference between coefficients.

TABLE II. Repeatability and Validity of Estimated Serum Cholesterol from Cholesterol per Unit Weight of Whole Blood Solids. All values in mg of cholesterol per 100 ml of serum.

$(S.E.M.)^2 = \Sigma\triangle^2/2N.$

Directly measured serum, mean	199.5
Estimated from whole blood, sample 1	197.3
Idem " 2	200.2
S.E.M. between samples of whole blood	±10.8
" " direct and whole blood estimate	±15.3

Repeated finger tip punctures. It is not suitable to take duplicate samples from the same finger prick unless an undesirably deep puncture is made. After taking first few drops as sample No. 1. a second sample requires "milking" the finger with resulting increased variability and lower average cholesterol concentration/unit of blood solids, presumably because of dilution with tissue fluid. Accordingly, comparisons were made between findings on bloods from 2 separate finger tip punctures on each of 13 men. An arm vein sample was obtained at about the same time. Data are summarized in Table II, serum cholesterol estimates from finger tip bloods being made by equation No. 10. Standard error of measurement, direct serum cholesterol *vs.* estimate from cholesterol/unit whole blood solids was ± 15.3 mg/100 ml. This agrees with corresponding value for the 93 sets of data used to obtain equation 10 (S.E.M. = ±15.6). S.E.M. for serum cholesterol values estimated from dried whole blood sample 1 *vs.* 2 was ±10.8 mg/100 ml and represents method error plus biological variation between bloods from different fingers punctured 10 minutes apart.

Discussion. Theoretically, estimation of serum cholesterol concentration from ratio of cholesterol to sodium in dried whole blood may be better than with whole blood as reference. The 95% range in normal man is only from 132 to 144 meq of sodium per liter of plasma, the average for blood cells is about 18 meq per liter of cells(9). Work on the method with sodium is in progress.

In screening and in epidemiological studies a major purpose for estimating cholesterol in

TABLE III. Intra-individual Variation in Serum Cholesterol Concentration, mg/100 ml. "No. pairs" = number of men except in series 2 where a total of 110 men provided 834 pairs of observations, each 1 to 2 weeks apart, over period of 3 years.
$(S.E.M.)^2 = \Sigma\triangle^2/2N$, where N = No. of pairs.

Series	Interval	Diet, etc.	Age	No. pairs	Chol., mg/100 ml Mean	S.E.M.
1	1-2 wk	Fixed	20-29	38	209.7	12.0
2	"		35-60	834	228.2	11.5
3	1 yr	*ad lib*	20-29	100	175.8	18.2
4	"		55-64	100	234.9	20.1

the blood is its possible use to identify risk of future coronary heart disease. In the Framingham Study of U.S. Public Health Service a cutting point of 260 mg of cholesterol per 100 ml was used(2). From a follow-up of 4 years it was reported that men with single sample values above 260 subsequently suffered 2.9 times the rate of new coronary disease experienced by men with cholesterol values under 260. In the Albany Study of N. Y. State Dept. of Health(3) a cutting point of 275 mg/100 ml was used. Men above that level had a coronary attack rate 3.4 times greater than men below that level. Both Albany and the Framingham data provide valid estimates of prognostic significance of single cholesterol measurements but they must be gross under-estimates of significance of true mean values. This follows from a consideration of intra-individual variability.

Table III summarizes data on intra-individual variability in serum cholesterol. Series 1 and 2(10) refer to samplings 1 to 2 weeks apart from men maintained throughout in a constant state with regard to amount and composition of diet, exercise, recreation and environment. Series 3 and 4 refer to samplings just one year apart (same season), from men of settled habits and occupation in same state of health (clinically healthy), and metabolic state (resting, fasting), on 2 occasions. Values in Series 3 and 4 must be under-estimates of average intra-individual variability in ordinary life over a period of one year.

For men living ordinary lives intra-individual variability over any appreciable period of time, in the directly measured serum cholesterol, is larger than same-day variability between estimate from finger tip whole blood and measured arm vein serum cholesterol. Besides suggesting that the dried blood estimate is relatively good, these data are important in evaluating such material as from Framingham and Albany. Since single blood samples are imperfect indicators of true mean cholesterol values of individuals, any classification of men above and below any cutting point on the basis of single blood samples will misclassify some men in regard to their true mean values. From distribution of individual (single sample) values in a group, and data as in Table III, frequency of misclassification above and below any selected cutting point may be estimated.

Elsewhere mathematical theory and application will be presented. Here it is enough to state that such analysis of Framingham and Albany data indicates that true individual mean values of serum cholesterol must show some 60 to 80% better separation of high risk from low risk men than reported.

Considerations of intra-individual variability indicate: 1) Real significance of serum cholesterol level as predictor of risk of coronary heart disease has been under-estimated in analyses reported heretofore. 2) Much improvement in reliability for prognosis of cholesterol measurements will be gained from a series of bloods taken from each individual at different times.

Summary. A method is presented for estimation of serum total cholesterol concentration from concentration of cholesterol/unit of whole blood solids, using a few unmeasured drops of finger tip blood dried in room air on filter paper. Prolonged storage at ordinary temperatures may elapse before analysis. In 124 sets of comparisons between se-

rum from arm vein and dried finger tip blood, standard error of measurement was ± 15.5 mg cholesterol/100 ml serum. This is comparable to intra-individual variability in direct serum measurement of blood samples drawn a few days apart and is smaller than variability between serum values in casual bloods drawn at longer intervals. Significance of blood cholesterol level in prediction of risk from coronary heart disease has been under-estimated in previous studies.

1. Technical Group and Committee on Lipoproteins and Atherosclerosis, *Circulation,* 1956, v14 (no. 4, part 2), 691.

2. Dawber, T. R., Moore, F. E., Mann, G. V., *Am. J. Pub. Health,* 1957, v47 (no. 4, part 2), 4.

3. Doyle, J. T., Heslin, A. S., Hilleboe, H. E., Formel, P. F., Korns, R. F., *ibid.,* 1957, v47, 25.

4. Rivin, A. V., Yoshino, J., Shickman, M., Schjeide, O. A., *J. Am. Med. Assn.,* 1958, v166, 2108.

5. Anderson, J. T., Keys, A., *Clin. Chem.,* 1956, v2, 145.

6. Abell, L. L., Levy, B. B., Brodie, B. B., Kendall, F. E., *J. Biol. Chem.,* 1952, v195, 357.

7. Snell, F. D., Snell, C. T., *Colorimetric Methods of Analysis,* Van Nostrand, New York, 1959, 3rd Ed v2, pp279-337.

8. Mancini, M., Keys, A., PROC. SOC. EXP. BIOL. AND MED., 1960, in press.

9. Albritton, E. C., editor, *Standard Values in Blood,* A. F. Tech. Report, No. 6039, Dayton, O., 1951.

10. Keys, A., Anderson, J. T., Grande, F., *Lancet,* 1957, vii, 959.

Received March 11, 1960. P.S.E.B.M., 1960, v104.

Erythropoietic Recovery Measured by Fe_{59} Uptake in Irradiated Mice Protected with Bone Marrow.* (25872)

E. A. MIRAND, J. G. HOFFMAN AND T. C. PRENTICE

Roswell Park Memorial Inst., and Physics Dept., University of Buffalo, N. Y.

Lethal effect of whole body irradiation of mice and other mammals(1,2) is offset by intravenous grafting of nonirradiated bone marrow which may be isologous, homologous, or heterologous. It is believed that the introduced marrow cells proliferate and provide recovery by cellular repopulation or by elaborating some humoral substance which stimulates recovery of indigenous marrow. It is possible too, that transfused marrow plays a beneficial, transitory function pending recovery of indigenous marrow. Survival of animal after lethal doses of radiation depends on time of hemopoietic recovery. This has been measured in mice by recovery of the count of circulating lymphocytes and granulocytes and by recovery of hemoglobin concentration and platelet count. Recovery of hemoglobin concentration is not a satisfactory index of recovery because it is obscured by the slow downward trend of circulating survivor cells over a period of 12 to 16 days after radiation (4). Odell and Caldwell(5) found donor type erythrocytes in irradiated rats in significant numbers only after 14 to 20 days. To examine quickly the recovery of erythropoiesis we used the Fe_{59} uptake method(6) and find that recovery begins after third day post-radiation and is discernible by sixth day when isologous cells are administered. Moreover, responses elicited by homologous and heterologous cells are measurably different from one another and from the isologous case by sixth day. The method appears to distinguish the 3 genetically different cell types by the induced Fe_{59} uptake.

Methods and materials. ICR/Ha Swiss male mice, 6 weeks old weighing 20-25 g were irradiated at target distance of 30 cm, 250 kv, filter ¼ mm Cu plus 1 mm Al, with a dose of 900 R. Survival was 50% at 6 days and 0% at 12 days. Protection was given with isologous bone marrow from 6-week-old cou-

* This study supported by grants from U. S. Atomic Energy Comm., and U. S. Public Health Service. Appreciation is extended to the following Summer Fellows: William Zenosky, Douglas King and Susan Getman.

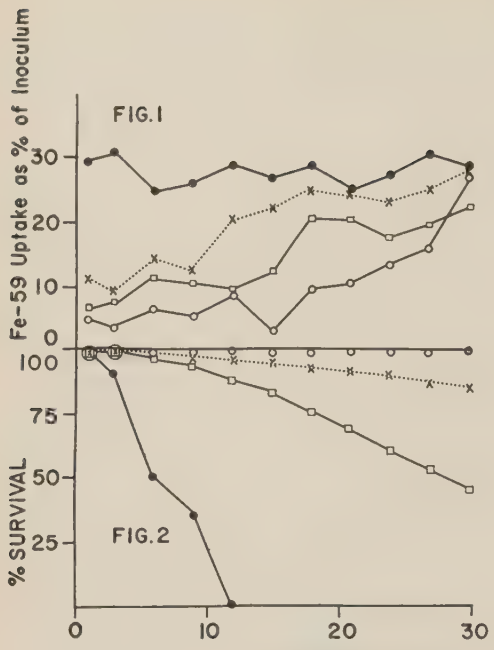


FIG. 3

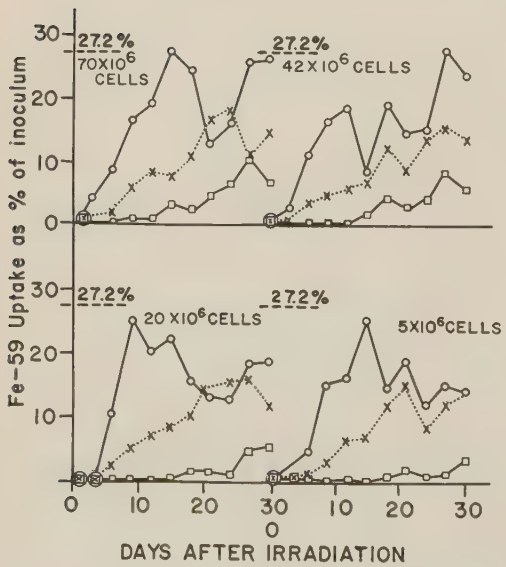


FIG. 1. Twenty-four hr Fe_{59} uptake in red blood cells of mice. Solid dots (●) show uptake in normal nonirradiated mice not receiving bone marrow cells. Other 3 curves show suppression of Fe_{59} uptake and subsequent recovery after 15 to 20 days in nonirradiated mice that received hypertransfusion of: ○ isologous, × homologous and □ heterologous marrow cells. Total cells administered were $(70 \pm 2) \times 10^6$, no suppression observed at 20 and 40×10^6 cell inocula. Cell numbers are averages. Each point represents a minimum of 6 mice and 6 determinations.

sin males; and with homologous bone marrow from DBA/1 male mice also 6 weeks old, and weighing 18-20 g. Heterologous bone marrow was taken from adult Sprague-Dawley rats (Holtzman) weighing 200-300 g. It should be emphasized that the term "isologous" in this report refers to the cousin breeding. Bone marrows were prepared by squeezing through filters the mashed tissue taken from femurs of donor mice and rats. Counts were made of viable, nucleated cells in Tyrode's Solution having 0.2% eosin. Cells stained with eosin were considered dead. Cell suspensions of known cell count were prepared and injected intravenously *via* the jugular vein within a period of 0 to 4 hours after whole body irradiation of the animal. Inoculum volume was kept constant at 0.2 cc. Assessment of erythropoietic activity was carried out as described elsewhere(6). On first, third, sixth and every third day thereafter to 30th day postradiation 6 mice were injected with 0.5 cc saline solution containing 1 μc of Fe_{59} *via* jugular vein. Uptake of Fe_{59} in circulating red blood cells was measured 24 hours after injection.

Results. Along with experimental groups of mice receiving bone marrow injections were 2 control series for each group. The first controls were mice having no radiation and no bone marrow administered. These controls totaling 156 mice established Fe_{59} uptake rate in normal mice. Solid dots in Fig. 1 give a representative series of uptake figures. From the 156 mice, average uptake rate was measured as 27.2%/24 hours, used as a basis for comparison in irradiated mice receiving mar-

FIG. 2. Survival rates of mice receiving 900 R whole body x-ray dose. Solid dots (●), mortality of mice not treated with marrow cells. Other curves show mortality in mice treated with: ×, homologous; and □, heterologous marrow cells. Circles (○) show mice receiving isologous cells had no mortality in 30 days. Cell dose was an avg of $(70 \pm 2) \times 10^6$ as in Fig. 1.

FIG. 3. Recovery of erythropoiesis as measured by Fe_{59} uptake in 24 hr interval in mice given 900 R whole body x-ray. ○, isologous; ×, homologous, and □, heterologous bone marrow cells administered intrav. postradiation. Number on each graph shows avg size of marrow cell dose. Coordinate at 27.2% uptake is avg uptake for normal nonirradiated mice not receiving marrow cells. Each point represents a minimum of 6 mice and 6 determinations.

TABLE I. Fe_{59} Uptake* as a Measure of Erythropoiesis in Swiss Male Mice Exposed to 900 R Whole Body X-Ray Dose. Uptake at various days postradiation. Marrow cells administered 0-3 hours postradiation.†

Marrow type	1 day	3 day	6 day	9 day	12 day
No marrow	.7 ± .1	.4 ± .2	.5 ± .1	.6 ± .2	.4 ± .1
Isologous	.4 ± .1	4.2 ±2.0	8.9 ±1.0	16.6 ±3.8	19.2 ±5.0
Homologous	.5 ± .2	.3 ± .1	1.9 ±3.2	5.9 ±1.0	8.3 ±1.9
Heterologous	.4 ± .1	.5 ± .3	.6 ± .3	1.0 ± .5	.8 ± .5

* Uptake in 24 hr expressed as % of inoculum.
† Cell doses were: isologous, 69 × 10^6; homologous, 71 × 10^6; heterologous, 72 × 10^6.

row cells for protection. This uptake rate in normal mice was in satisfactory agreement with earlier data(6) on Swiss male mice. No mice in this series of controls died.

The extent to which addition of marrow cells to normal, nonirradiated mice would depress normal erythropoiesis was determined in second series of controls. From earlier runs (6) it was known that hypertransfusion of red blood cells suppressed erythropoiesis as measured by Fe_{59} uptake. In 3 series of 54, 60 and 60 nonirradiated mice, each mouse received an average of 70 x 10^6 marrow cells respectively of isologous, homologous and heterologous type. Fig. 1 shows effect on Fe_{59} uptake percentages. There is a depression in the first 15 days postradiation and a subsequent recovery. The greatest depression and longest time of recovery occurred when isologous marrow was administered. There was no mortality in these controls. When 23 x 10^6 and 44 x 10^6 bone marrow cells were inoculated no depression in Fe_{59} was observed.

Protection against lethal effect of 900 R was based on previous determination of mortality(6) at this x-ray dose level. A series of 40 mice receiving x-ray without marrow established that by 12 days postradiation all animals died of severe anemia, septicemia hemorrhage and in some instances of gastrointestinal injury. Fifty percent survival was at 6 days (Fig. 2, solid dots). Measurements of Fe_{59} uptake rate are given in first line of Table I. Figures range from .7% to .4% and are much lower than those for normal or hypertransfused mice(6). The lethal effect of radiation appears to be measured by failure of Fe_{59} uptake rate since normal rates of uptake are in the range of 25 to 30%, (Fig. 1).

Survival data on irradiated mice given an average of 70 x 10^6 marrow cells of the 3 different types are shown in Fig. 2. Those receiving isologous marrow cells survived 100% after 30 days whereas homologous and heterologous cells conferred lower survivals. Homologous cells gave an 83% and heterologous cells a 45% survival after 30 days postradiation. The question as to degree of protection afforded by numbers of marrow cells was examined by administering various average numbers of cells ranging from 5 to 70 x 10^6 (Table II). Isologous cells in doses 5, 20, 40 and 70 x 10^6 afforded 100% survival at 30 days. Also, varying the cell numbers by a factor of 14 produced no significant change in survival at 30 days when homologous or heterologous cells were given. Thus, homologous cells in all doses permitted about 82% survival and heterologous cells led to a 55% survival although number of cells injected varied from 5 to 70 x 10^6. The lack of any big variation in survival with change of inoculum size suggests that there may be a threshold effect wherein a minimum number

TABLE II. Survival Data on Swiss Male Mice Given 900 R Whole Body X-Ray Dose and Treated with Bone Marrow Cells Intravenously. % survival after 30 days as function of average number of marrow cells given.

Marrow type*	5 × 10^6 cells		20 × 10^6 cells		40 × 10^6 cells		70 × 10^6 cells	
		%		%		%		%
Isologous	(59)	100	(58)	100	(59)	100	(54)	100
Homologous	(64)	81	(66)	84	(63)	81	(60)	83
Heterologous	(66)	64	(60)	61	(59)	49	(66)	45

* 40 mice given no marrow were all dead by 15th day. Parentheses contain total No. of mice used in determination of survival rates.

of cells suffices to induce the protective processes.

Discussion. The Fe_{59} uptake method for detecting hemopoiesis gives an early postradiation indication of erythropoiesis. The earliest manifestations of erythropoiesis reported hitherto are those of Odell and Caldwell(5) and deVries and Vos(7) who showed that in rats donor erythrocytes became dominant beginning at 14 days after marrow injection. Referring to Fig. 3 the 5 x 10^6 cell doses of isologous, homologous and heterologous produced responses in uptake rate detectable at 3 to 6 days postradiation. At 6 days, uptake for isologous cell treatment was 8.9% and those for homologous and heterologous cells were 1.2 and .5% respectively. Table I gives more detailed figures for responses at the 70 x 10^6 cell dose levels. Mice without marrow cell treatment had an uptake less than 1% as pointed out above (data in Table I, first line).

The isologous cells produced an uptake of 4.2% by the 3rd day, a figure readily measurable. In this respect, the Fe_{59} uptake method compared favorably with the lymphocyte count method(4) which is sensitive since these cell types of the recipient are removed quickly from circulation after radiation. These results are consistent with the view that in supralethally irradiated mice the isologous cells provide erythropoiesis. Uptake of Fe_{59} thus gives a sensitive and quick measure of erythropoiesis in presence of high levels of hemoglobin concentration at early times postradiation.

The significant differences seen between the curves for various injected groups points out that isologous bone marrow populates sooner than homologous and heterologous bone marrow. Factors for this are not definitely understood, however, isologous bone marrow certainly stands a better chance for cellular repopulation in a more genetically related irradiated host.

The mortality data do not correlate closely with the data on erythropoietic activity as measured by Fe_{59} uptake rate, (Fig. 3). The ordinate at 27.2% shows normal uptake in nonirradiated mice without marrow cell injection. Uptake in mice given isologous cells shows a rapid return to normal rates in all cases, by the 15th day, which is independent of cell numbers administered. This is also the result for mice given homologous cells, except that where the lowest cell dose was given (5 x 10^6 cells) initial recovery in the first 9 days is slightly slower than at higher cell number doses. Mice receiving less than 40 x 10^6 heterologous cells have a much lower recovery of uptake than do those receiving doses larger than 40 x 10^6, (Fig. 3). The difference, however, did not appear to affect survival figures, (Table II). It is, indeed, hard to explain the 64% survival rate, (Table II), on the basis of uptake data for a cell dose of 5 x 10^6.

The mortality data of Table II bear out the established genetic relationship between donor and recipient described by Trentin(2) and Uphoff and Law(8). The heterologous marrow cells being farther removed genetically from the recipient afforded less survival. The trends of the curves in Fig. 3 fit the concept that isologous cells are genetically closer to the recipient than are homologous cells from DBA/1 mice or the heterologous cells from rats. Iron uptake rates are clearly greatest for the isologous cells: homologous cells stimulate erythropoiesis greater than do heterologous cells but less than isologous cells. The consistency of genetic behavior as cell type varies supports the inference that protection conferred by the marrow cells is not due to stimulation of erythropoiesis alone. This is clearly indicated in the heterologous case wherein the Fe_{59} uptake is very low for the first 15 days postradiation. Hence survival cannot be due to erythropoiesis alone. If protection depends on cellular elements in circulation then "the reticulo-endothelial and myelogenous systems" apparently are involved in protection afforded by the marrow graft. For example, the early appearance of circulating leucocytes as shown by Odell and Caldwell(5), and the effect of cell and spleen extracts, as shown by Goldfeder and Clarke (9), suggest other supporting mechanisms necessary for survival.

Summary. 24 hour uptake of Fe_{59} was

used as quick measure of erythropoiesis in Swiss male mice. In normal nonirradiated mice, iron uptake is not affected by intravenous injection of isologous, homologous and heterologous bone marrow cells except for inocula greater than 40 x 10^6 cells. Injection of these cell types after 900 R whole body x-radiation increases Fe_{59} uptake rates in the order: heterologous, homologous, isologous. Protection against lethal radiation effect as measured by survival and Fe_{59} uptake depended on genetic relationship between bone marrow donor and recipient.

1. Doherty, D. G., Congdon, C. C., Makinodan, T., Hollaender, A., *Radiation, Biology and Medicine,* Reading, Mass., 1958, Chap. 15, 369.

2. Trentin, J. J., *J. Nat. Cancer Inst.,* 1959, v22, 219.

3. Gengozian, N., Makinodan, T., Congdon, C. C., Owen, R. D., *Proc. Nat. Acad. Sci.,* 1958, v44, 560.

4. Smith, W. W., Marston, R. Q., Cornfield, J., *Blood,* 1959, v14, 737.

5. Odell, T. T., Jr., Caldwell, B. C., *J. Nat. Cancer Inst.,* 1958, v20, 851.

6. Mirand, E. A., Hoffman, J. G., Prentice, T. C., Reinhard, M. C., *Exp. Med. Surg.,* 1958, v16, 258.

7. de Vries, M. J., Vos, O., *J. Nat. Cancer Inst.,* 1959, v23, 1403.

8. Uphoff, D. E., Law, L. W., *ibid.,* 1959, v22, 229.

9. Goldfeder, A., Clarke, G. E., *Rad. Res.,* 1957, v6, 318.

Received April 4, 1960. P.S.E.B.M., 1960, v104.

Effects of Pyridoxine and Desoxypyridoxine on Magnesium Metabolism in the Rabbit.* (25873)

JERRY K. AIKAWA
(With technical assistance of Jacqueline Z. Reardon and Dale R. Harms)
Dept. of Medicine, University of Colorado School of Medicine, Denver.

Factors regulating metabolism of magnesium are obscure. Development of a relatively simple and reliable chemical method for determination of magnesium in biological material, and availability of radioactive isotope of magnesium, Mg^{28}, have made possible a restudy of the entire problem of magnesium metabolism. Studies of plasma clearance and tissue uptake of Mg^{28} in experimental animals (1) and in human beings(2) have been reported. Changes in carbohydrate metabolism alter rate of uptake of Mg^{28} by various tissues(3). The effects of cortisone acetate have also been determined (unpublished). These studies suggested that tissue uptake of Mg^{28} was considerably slower than that of another intracellular ion, potassium, and that cellular uptake of magnesium might be associated with anabolic activities of cells. If this hypothesis is correct, then any factor altering metabolism of protein might affect that of magnesium. Pyridoxine deficiency alters protein metabolism and produces changes in enzymatic reactions(4); pyridoxine also influences magnesium metabolism(5). Our experiments were undertaken to determine effects of pyridoxine and its antagonist, desoxypyridoxine, on magnesium metabolism in the rabbit. Mg^{28} was used to measure exchangeable body content of this ion and its relative tissue uptake.

Material and methods. Forty-seven normal adult male rabbits weighing between 1.6 and 2.5 kg were kept in individual stainless steel metabolism cages and fed a stock diet of compressed pellets. Tap water was given *ad lib.* *Pyridoxine* hydrochloride and *desoxypyridoxine* hydrochloride were dissolved in distilled water, neutralized with 1 N NaOH, and made to concentration of 40 mg/ml. *Exp. I and II.* External balance studies for magnesium were performed daily at least 11 days in 2 groups of 8 rabbits each. During first 2 days, baseline studies were done. Starting on third day and continuing 8 days, each animal in

* This work supported by contract between University and U. S. Atomic Energy Comm. and by grant from Colorado Heart Assn.

Exp. I received daily dose of *pyridoxine* (100 mg), given intravenously into marginal ear vein. Starting on third day and continuing 6 days, each animal in *Exp. II* received daily dose of *desoxypyridoxine* (40 mg/kg) given by same route. *External balance of magnesium* and *exchangeable magnesium content* (Mg_e) were determined as previously described(1). Magnesium concentrations in urine and serum were determined by modification of the molybdivanadate method for phosphate(6). *Exp. III and IV.* The remaining 31 animals were divided into 3 groups. Each of 8 rabbits in *Exp. III* received intravenous dose of *pyridoxine* (100 mg). Twenty-four hours later, a second dose of pyridoxine and 5 ml of Mg^{28} solution, used in Exp. I and II, were given intravenously. Animals were killed by air embolism exactly 4 hours after injection of Mg^{28}, and samples of tissues obtained. Tissue relative activity was determined as previously described(1). Each of 15 rabbits in *Exp. IV* received 7 daily doses of *desoxypyridoxine* (40 mg/kg) given intravenously. The seventh injection of desoxypyridoxine was accompanied by injection of 5 ml of Mg^{28} solution. Animals were then killed by air embolism at 4 hours, and tissues obtained as described previously(1). Eight animals in the *control group* were not given pyridoxine or desoxypyridoxine, but were otherwise treated identically with those in test groups. *Exp. I and II.* Each animal served as its own control. After the difference between baseline values and values at end of treatment period had been calculated for each animal, mean differences for the whole group were obtained. The significance of mean differences was determined by use of "t" test(7), a "P" value of less than 0.01 being considered significant. *Exp. III and IV.* Mean relative activity of each type of tissue was determined for both test groups and for control group, and the significance of differences between group means was tested(7).

Results. Exp. I and II. Baseline values for serum magnesium, exchangeable magnesium, Mg^{28} excretion, food intake, urinary magnesium excretion, urine volume and magnesium balance were similar to those previously reported in other similar experiments (3). *Exp. I.* Clinically, animals injected with *pyridoxine* appeared healthy throughout experiment. With administration of pyridoxine, a significant increase in mean body weight (2.195 to 2.272 kg) occurred. No other significant changes were noted in this external balance study. Exchangeable magnesium content was not altered. *Exp. II.* After third injection of *desoxypyridoxine,* rabbits became hyperirritable. About 2 hours after fifth and sixth injections of the drug, generalized convulsions occurred, lasting several minutes. No significant changes were noted in mean body weight or in other parameters studied.

Exp. III and IV (Table I). In rabbits given *pyridoxine,* a significant increase was observed in relative tissue activity of appendix and heart. Rabbits given *desoxypyridoxine* showed a significant increase in serum magnesium concentration and a significant de-

TABLE I. Effect of Pyridoxine, 100 mg Daily for 2 Days, and Desoxypyridoxine, 40 mg/kg Daily for 7 Days, on Relative Radioactivity* of Tissues in Rabbits.

Tissue	Control group[1]	Pyridoxine group[2]	Desoxy-pyridoxine group[3]
	Relative radioactivity*		
Muscle	.50 ± .10†	.78 ± .12	.58 ± .06
Skin	1.25 ± .14	1.83 ± .25	1.43 ± .12
Appendix	3.90 ± .23	8.26 ± .61‡	5.57 ± .30
Lung	4.25 ± .26	4.52 ± .29	3.19 ± .12‡
Liver	5.10 ± .42	7.10 ± .61	4.65 ± .28
Heart	5.90 ± .99	10.37 ±1.03‡	6.85 ± .35
Kidney	11.00 ± .60	11.42 ± .63	8.65 ± .48‡
Bone	12.90 ±1.13	16.54 ±1.25	9.29 ± .50‡
	Mg concentration		
Serum (meq/l)	2.06 ± .14	1.93 ± .03	2.80 ± .28‡
	Blood glucose (mg %)		
	91.4 ±5.27	102.8 ±6.22	92.9 ±4.01

* Relative radioactivity $= \frac{\text{cpm/g tissue}}{\text{cpm/ml serum}}$ at time of death.

† Stand. error.

‡ Statistically significant difference when compared with control group.

[1] Mean value in 8 rabbits not inj.

[2] Mean value in 8 rabbits receiving 2 daily inj. of pyridoxine.

[3] Mean value in 15 rabbits receiving 7 daily inj. of desoxypyridoxine.

crease in relative tissue activity of kidney, lung and bone.

Comment. In previous experiments with rabbits, administration of insulin and glucose increased relative tissue activity of all tissues studied(3). Cortisone increased exchangeable magnesium content of body and uptake of Mg^{28} in muscle, appendix and heart. The increase in exchangeable magnesium was attributed primarily to increased uptake of Mg^{28} in muscle, since the relative mass of appendix and heart is too small to have an appreciable influence on exchangeable magnesium content of the body.

Under our conditions, pyridoxine did not alter clinical behavior of rabbits, although it may have accelerated body growth. It did not affect the external balance of magnesium, and exchangeable body pool of magnesium was not altered. Relative tissue uptake of Mg^{28} was increased in appendix and heart only, and not in muscle. These observations are consonant with the impression that exchangeable magnesium content of body is increased by conditions which increase uptake of Mg^{28} by muscle and bone, the body's largest reservoirs of magnesium.

The dose of desoxypyridoxine administered was sufficient to produce typical convulsions of pyridoxine deficiency. Animals receiving desoxypyridoxine, however, showed no significant changes in external balance of magnesium, and the body's pool of exchangeable magnesium was not altered. Uptake of Mg^{28} in kidney, lung, and bone was suppressed, and serum magnesium concentrations were elevated. Elevation of serum magnesium, in presence of unaltered urinary excretion of magnesium, suggests that renal excretion of magnesium was not suppressed by desoxypyridoxine; it further suggests that pyridoxine deficiency might alter distribution of magnesium between intra- and extra-cellular compartments.

Summary. External balance studies for magnesium and isotopic determinations of exchangeable magnesium content of body were made in 8 adult male rabbits before and after 8 daily injections of pyridoxine (100 mg), and in another group of 8 rabbits before and after 6 daily injections of desoxypyridoxine, 40 mg/kg. Except for increase in mean body weight in the group receiving pyridoxine, no significant changes were observed. In 8 animals given 2 intravenous injections of pyridoxine (100 mg) 24 hours apart, relative tissue activity of Mg^{28} was increased in appendix and heart. In 15 rabbits given desoxypyridoxine, 40 mg/kg, daily for 7 days, there was a significant decrease in relative activity of kidney, lung, and bone, and a significant increase in serum magnesium concentration. The results suggest that excess or deficiency of pyridoxine alters rate of uptake of Mg^{28} by various tissues.

Mg^{28} was supplied by Brookhaven National Lab. on allocation from U. S. Atomic Energy Comm.

1. Aikawa, J. K., Rhoades, E. L., Harms, D. L., Reardon, J. Z., *Am. J. Physiol.,* 1959, v197, 99.

2. Aikawa, J. K., Gordon, G. S., Rhoades, E. L., *J. Applied Physiol.*

3. Aikawa, J. K., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 363.

4. Meister, A., *Advances in Enzymol.,* 1955, v16, 185.

5. Buscaino, V. M., Balbi, R., *Acta neurol. psychiat. belg.,* 1952, v7, 1.

6. Aikawa, J. K., Rhoades, E. L., *Am. J. Clin. Path.,* 1959, v31, 314.

7. Snedecor, G. W., *Statistical Methods,* Iowa State College Press, 1946.

Received April 6, 1960. P.S.E.B.M., 1960, v104.

Action of Glycine and a Lysozyme-like Agent from Rabbit Monocytes in Destruction of Brucella.* (25874)

D. J. Ralston and S. S. Elberg

Dept. of Bacteriology, University of California, Berkeley, and Lab. of Comparative Biology, Kaiser Fn. Research Inst., Richmond, Calif.

Results of studies carried out with vaccine strain of *Brucella melitensis* Rev. Is suggest that death of these bacteria may be produced in stepwise fashion by action of enzyme on its wall, but only after the substrate has been made available by an agent which causes preliminary damage. This study summarizes experiments which indicate that normal rabbit monocytes contain a lysozyme-like material capable of attacking Brucella cells. Used alone, this agent exhibits no inhibition of living Brucella, presumably because the specific configuration of cell wall prevents its action. When the enzyme is combined with the amino acid, glycine, death and lysis take place. Evidence is presented to indicate that a similar reaction may take place intracellularly, in monocytes parasitized with Brucella, when tissue culture medium is supplemented with sufficient amounts of glycine. These studies place emphasis on the possible role of similar agents, which, under physiological conditions existing in intact animal, may contribute to inhibition of growth and ultimate disposal of organisms responsible for Brucellosis.

Methods. Bacterial cultures. Smooth cultures of *B. melitensis* Rev Is(1) were maintained on Albimi Brucella agar slants. For tests of enzyme action on killed cells, growth was harvested after 3 days at 37°C into saline or Zobell's solution ($K_2HPO_4 \cdot 3H_2O$, 1.31 g; KH_2PO_4, 0.75 g; NaCl 2.5 g; $MgSO_4 \cdot 7H_2O$, 0.10 g, $CaCl_2 \cdot 2H_2O$, 0.0056 g; Cysteine-HCl, 0.10 g, H_2O, distilled, 1 liter; pH 7.0-7.4). Suspensions containing approximately 10^{10} bacteria/ml were heated 2 hr at 60°C, washed in saline, centrifuged, and the pellets treated with N-butanol at room temperature for ½ hr and sometimes overnight at 4°C. Butanol was removed; cells were resuspended in saline and stored in the cold. For tests on living Brucella, cells were harvested either from shaken cultures in Albimi broth at 37°C or from young growth on Albimi agar (14-24 hrs). For infection of monocytes, cells were harvested after 1-3 days on Albimi agar, suspended in Tyrode's solution to approximately 10^9/ml, and diluted in tissue culture medium. *Preparation of normal rabbit monocytes.* Peritoneal exudates were collected 5 days after injection of 40-50 ml Klearol into 3-5 lb rabbits. Previous experiments have shown that such exudates contain 85% or more monocytes(2). The peritoneal cavities were washed with either saline or Tyrode's, and exudate cells were aspirated through sterile cheese cloth filters into separatory funnels. The oil was discarded; cells were centrifuged 10 minutes at approximately 250 g in the cold, washed once in saline or Tyrode's, counted in Petroff Hausser Chamber, and used for extraction or for monocyte infection experiments. In the latter case, cells were harvested in Tyrode's solution. In general, extreme washing was avoided, as was treatment with proteolytic enzymes, to reduce chances of removing active factors. *Monocyte maintenance medium.* Parasitization of monocytes and maintenance was carried out in medium composed of 40% fresh, unfiltered, normal rabbit serum and 60% Tyrode's, made as follows: NaCl, 7.7 g; KCl, 0.2 g; NaH_2PO_4, 0.05 g; $MgSO_4$, 0.1 g; Dextrose, 4 g; phenol red, 1 ml of 1% solution; distilled water, 990 ml. After autoclaving, sterile $NaHCO_3$ was added to final concentration of 0.5 g and $CaCl_2$ to final concentration of 0.14 g. When Tyrode's was used for harvesting monocytes, calcium was omitted. pH was adjusted to 7.0-7.4 with sterile H_3PO_4. Monocyte cultures were set up in 25 ml Erlenmeyers layered with 2 to 3 ml medium containing parasitized monocytes. On some occasions solutions were distributed into 12 ml

* This research supported by grant of Nat. Inst. Health, and funds from Office of Naval Research.

TABLE I. Effect of Monocyte Extracts from Normal and Immune Rabbits on Heat-Killed, Butanol-Extracted Brucella.

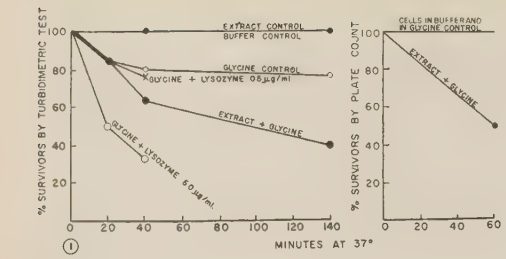
Source	Dilution of extract	% lysis, 18 hr 37°	Viscosity	Reaction with Alcian Blue	Residual cells remaining after treatment with extract: % lysis upon addition of crystalline lysozyme, 40 μg/ml	Viscosity
Immune	1/40	21	+	+	0	—
	1/80	25	+	+	0	—
	1/160	24	+	+	0	—
	Control	6	—	—	24	+
Normal	1/40	25	+	+	not tested	not tested
	1/80	24	+	+	*idem*	*idem*
	1/160	24	+	+	"	"

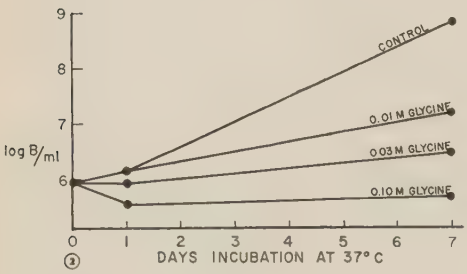
conical centrifuge tubes and incubated slantwise. Viability of monocytes was ascertained by staining suspensions with eosin, after method of Hanks(3).

Results. Preparation and properties of a lysozyme-like agent from rabbit monocytes. The agent may be extracted from normal and immune rabbit monocytes by procedures whereby the cells are broken open by freeze-thaw or Mickle disintegration technics. Usually active material has been obtained from extracts prepared by freeze-thawing 2 to 5 $\times$ 10^8 monocytes in 5 to 10 ml N/1 or N/10 acetic acid solution, pH 3.6, mixed with equal parts of physiological saline, as used by Amano, *et al.*(4); by freeze-thawing of monocytes that had been stored in Tyrode's solution for 1-2 weeks at 4°C; or by freeze-thawing in "intracellular salts" solution, according to procedure of Hirsch(5). An active material has also been obtained by Mickle disintegration in phosphate buffer at pH 7.0 or in distilled water, but these preparations lose activity on storage. Extracts were adjusted to pH 6.8 to 7.0 with NaOH; precipitates and debris were removed by centrifugation. Yields from 2-3 $\times$ 10^8 monocytes exhibited lysozyme activity for *M. lysodeikticus* comparable to concentrations of crystalline egg white lysozyme (Worthington) in the order of 500 μg.

Action on Brucella cells. Heat-killed and Butanol-, Acetone-, or Chloroform-Extracted. Cells suspended in phosphate buffer at pH 8.0 on treatment with the extract undergo reduction in turbidity (Table I); the extent varies with the particular cell preparation. This is accompanied by release of a viscous material, which is destroyed by crystalline DNAse, indicating it is bacterial DNA. Crystalline lysozyme causes a similar reaction. Trypsin, chymotrypsin, and pancreatic lipase also cause reduction in turbidity, but, in these cases, cause no release of viscous material. When washed out of the monocyte extract or lysozyme reaction mixture and resuspended in distilled water, the cell residue is not osmotically fragile, in contrast to results obtained with living cells. Table I also indicates that extracts prepared from exudates produced in Rev Is immunized rabbits contain comparable amounts of the agent. The viscous material released from cells reacts with Alcian Blue dye to form a stringy precipitate.

Action on living Brucella cells. The material in monocyte extracts has no killing or lytic effect when tested alone in living cells, either in Albimi media, or in phosphate or veronal buffers, pH 7.0 to 8.0. When combined with glycine, an accelerated lysis and death takes place (Fig. 1). This effect takes place more rapidly with fast growing, young cells, than with stationary phase preparations. Rough cells appear to be more susceptible than smooth, and tests with a virulent strain *B. melitensis* indicate that cells of a comparable age are more resistant than either rough or smooth Rev Is. With living cells, the monocyte extract also brings about a release of bacterial DNA; lysozyme produces a similar effect. This response is not observed with the following enzymes: pancreatic lipase, trypsin, chymotrypsin, RNAse, DNAse or





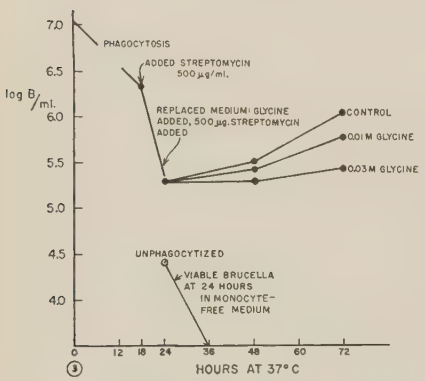


FIG. 1. Effect of glycine, monocyte extract or lysozyme, and their combination on smooth cells of Rev Is, in buffer-Tyrode's solution, at pH 6.2.

FIG. 2. Intracellular brucella in normal rabbit monocytes treated with varying amounts of glycine, in Tyrode's serum medium.

FIG. 3. Intracellular multiplication of Rev Is in rabbit monocytes treated with varying concentrations of glycine in presence of 500 µg/ml streptomycin.

D-amino acid oxidase. This suggests that the monocytic agent is a cellular lysozyme. Other properties which suggest its lysozyme-like nature include: (1) its resistance to trypsin, (2) heat stability, (3) action on *M. lysodeikticus,* and (4) its action on cell wall preparations of Brucella, *Micrococcus lysodeikticus,* and *S. aureus,* all containing the mucopolysaccharide substrate for lysozyme. Further details of these studies will be published.

Action of glycine on growth of Rev Is in monocytes in tissue culture medium. To determine whether glycine might also act together with the lysozyme-like agent in parasitized monocytes, a suspension of 1×10^6/ml of these cells was exposed to viable bacteria, and, after phagocytosis, the infected monocytes were washed and resuspended in fresh medium, to which was added varying concentrations of glycine, ranging from 0.01 to 0.3 M (Fig. 2). In one set of cultures, streptomycin was included to prevent extracellular multiplication of bacteria. Fig. 3 shows effects of streptomycin treatment on yields of intracellular bacteria. The data suggest that glycine exerts some action on the monocyte culture, in that growth of bacteria is reduced in presence of glycine. In other experiments, addition of 0.03 M glycine completely prevented bacterial multiplication in parasitized monocytes. Examination of stained preparations revealed extensive multiplication in untreated control cells. Concentrations of glycine used exerted no deleterious effects on monocytes themselves.

Discussion. These results suggest that extracts of monocytes contain an agent which resembles a lysozyme. The living Brucella is very resistant to the agent, but, when treated with glycine, a damage occurs which then allows the extract to lyse the cells. This indicates that, within the living monocyte, the agent can operate only when a similar damage takes place. It is possible that glycine plays a role similar to versene in that it chelates essential metal ions and renders the Gram-negative wall susceptible to lysozyme, in a manner observed with other bacteria(6). On the other hand, it may act by disturbing wall synthesis, thereby exposing the substrate in a form accessible to the agent. Glycine has been observed to produce protoplast-like structures of Brucella by Gerhardt(7) perhaps by this mechanism. Gary, *et al.*(8), working with *B. abortus* reported that addition of amino acid supplements, especially containing DL-valine and L-lysine, tend to inhibit total polysaccharide synthesis in growth media containing glucose, yeast autolysate, and casein hydrolysate. The work of Shockman, *et al.*(9), on conditions leading to cell lysis of *S. faecalis,* also suggests that the

physiological balance of growth medium may lead to imbalance of wall synthesis and cell lysis. Related effects may be occurring in those intracellular systems which prevent multiplication of Brucella, thereby rendering the bacteria more accessible to intracellular enzymes. The results of these experiments suggest that *in vitro* attempts to reconstitute intracellular conditions must take into account the necessity for establishing the proper physiological test environment; that death of cells may be a stepwise series of reactions; and that omission of any of the necessary components may result in failure to demonstrate the factors concerned. Operation of a mechanism similar to that produced by glycine in monocytes may be involved in natural resistance to infection. It is non-specific and perhaps dependent upon day-to-day variations in physiological levels of at least 2 agents.

Summary. A lysozyme-like material from rabbit monocytes is described. It acts on glycine-treated brucella to cause lysis and death. When parasitized monocytes containing this agent are treated with low concentrations of glycine, intracellular growth and yields of smooth *Brucella melitensis,* Rev Is is suppressed. The theory is proposed that intraphagocytic death involves a stepwise alteration of the wall, followed by enzymatic degradation of the mucopolysaccharide component responsible for structural integrity of the bacteria.

1. Elberg, S. S., Faunce, K., *J. Bact.,* 1957, v73, 221.

2. Fong, J., Schneider, P., Elberg, S. S., *J. Exp. Med.,* 1956, v104, 455.

3. Hanks, J. H., Wallace, J. H., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 188.

4. Amano, T., Inai, S., Seki, Y., Kashiba, S., Fujikawa, K., Nishimura, S., *Med. J. Osaka U.,* 1954, v4, 401.

5. Hirsch, J. G., *J. Exp. Med.,* 1956, v103, 589.

6. Repaske, R., *Biochim. Biophys. Acta,* 1956, v22, 189.

7. Gerhardt, P., *Bact. Rev.,* 1958, v22, 81.

8. Gary, N. D., Kupferberg, L. L., Anderson, R. E., Harein, P. K., Klausmeier, R. E., *J. Bact.,* 1957, v73, 632.

9. Shockman, G. D., Kolb, J. J., Toennies, G., *J. Biol. Chem.,* 1958, v230, 961.

Received April 11, 1960. P.S.E.B.M., 1960, v104.

Effect of Metabolic "Stress" on Development of Tumor Following Inoculation of Walker Carcinosarcoma Cells.* (25875)

JOHN D. GRIFFITHS† AND EVERETT HOPPE (Introduced by W. H. Cole)
Dept. of Surgery, University of Illinois College of Medicine, Chicago, Ill.

The effect of dietary deficiency in production of spontaneous tumors has been well described by Tannenbaum and Silverstone(1) who demonstrated that prolonged restricted caloric intake and malnutrition in laboratory animals will diminish induction and growth of spontaneous tumors. However, to our knowledge the influence of acute metabolic changes has not been investigated in relationship to increased susceptibility of rats to inoculation of transmissible malignant tumor cells. In this present investigation the effect of "acute" metabolic changes, such as starvation and dehydration, which can be considered a form of metabolic "stress," have been studied in relation to the animals' susceptibility to inoculation of Walker 256 carcinosarcoma.

* Supported in part by grant from USPHS.

† Eli Lilly Educational Fellow.

Method. Female Holtzman white rats weighing between 215 and 230 g were divided into 5 groups and housed in the same room with identical diet (Purina lab-chow) for 3 weeks. They were weighed every day to be certain a similar weight gain was obtained in all animals before experiment was started. At end of this period, the groups were treated as follows: In *Group 1* water bottles were removed for 48 hours but "chow" feed was still available; in *Group 2* food was taken away

TABLE I. Effects of Dehydration and Starvation on "Takes" of Walker 256 Carcinosarcoma in Rats.

	No. of rats*	% "Take"	Loss or gain in wt
1. Dehydration, 48 hr	43	77%	Loss, 39 g
Control	23	60%	Gain, 5 g
2. Complete starvation, 48 hr	75	89%	Loss, 35 g
Control	50	76%	Gain, 3 g
3. Starvation and dehydration, 48 hr	21	69%	Loss, 35 g
Control	21	71%	Gain, 3 g
4. Starvation, 7 days	44	80%	Loss, 50 g
Control	42	57%	Gain, 12 g

* 25,000 cells ("aged" 12 hr to reduce "takes" below 100%) inoculated subcut. over the abdomen —half of each group at commencement of experiment; the other half at end of deprivation. There was no significant difference between the 2 groups.

for 48 hours but water bottles were left; in *Group 3* animals were deprived of food and water for 48 hours; *Group 4* was kept without food for 7 days but water was still available. The *5th Group* was fed and watered in the normal way and used as control animals. A suspension of Walker 256 carcinosarcoma was made as described by Talalay *et al.*(2) and "aged" (Chan *et al.*) to diminish virulence of tumor suspension, and thus produce a lower percentage of "takes." A suspension containing 25,000 cells aged 12 hours was injected subcutaneously over the abdomens of half of each group at commencement of the period of experiment and the other half at the end of period of deprivation. Control groups of rats were also inoculated at the same time as experimental groups with the same tumor suspension and same number of cells. Following period of deprivation of food and water the rats were fed the identical diet given previous to experiment. Tumors developed in 14-21 days in rats in which "takes" occurred. The rats in which tumors did not develop were kept for 3 months to safeguard against late development of tumors, but none developed. The effect of "takes" in rats during growth periods was investigated in 5 groups of female Holtzman rats of ages 28 to 150 days, when they can be considered mature; all rats were inoculated at the same time with the same suspension of "aged" Walker 256 carcinosarcoma cells subcutaneously on their abdomens. The rats were then kept under same conditions and fed same diet. At end of 3 weeks number of "takes" was counted and rats in which no tumor developed were observed for 3 months.

Results. Table I indicates that acute dehydration and acute starvation of 48 hours duration did not influence the "take" of Walker cells inoculated subcutaneously.

However, following starvation for 7 days, 80% of 44 rats developed tumors, compared to 57% of 42 control rats ($\chi^2 = 5.007$). There was no mortality during period of starvation or dehydration. Weight charts were kept on each group of rats for 24 days before and 10 days after starvation or dehydration (Table I).

The "takes" of Walker tumor cells inoculated subcutaneously into rats of varying ages (Table II) show no difference in the 4 groups varying between 145 and 250 g in weight. However, the incidence of "takes" in the young group with average weight of 85 g was considerably higher than the mature rats (94% vs. 68 to 73% ($\chi^2 = 4.97$)).

Discussion. Starvation and dehydration produce a disturbance in metabolism which might be considered a metabolic "stress." Anabolism is replaced by catabolism and there is a mobilization of body's reserves. Neither starvation nor dehydration nor combination of both for 48 hours produced any increase in susceptibility of animal to subcutaneous inoculation of Walker tumor. However, following prolonged starvation for 7 days, when there had been a loss of one-fifth of body weight, there was an increase in per-

TABLE II. Effect of Growth and Weight Changes on "Takes" of Walker 256 Carcinosarcoma in Rats.

Age (days)	Wt (g)	No. of rats*	% "Takes"
28	85	19	94
60	145	21	71
80	160	19	73
110	210	18	66
Mature	250	22	68

* 25,000 cells "aged" 12 hr were inoculated on same day in 5 groups of rats, of different age groups, using same suspension.

centage "takes" of experimental animals over normal control group. This leads to the belief that following marked catabolic stress conditions in tissues of experimental animal are more receptive to the implanted malignant cell than tissues of normal healthy animal.

At first glance the increased resistance of Tannenbaum and Silverstone's animals with chronic malnutrition to development of spontaneous tumors might appear to be at variance with our data showing that acute starvation reduces resistance of the rat to inoculated Walker 256 cells. However, in our estimation there is a marked difference in metabolic status of the 2 groups of animals. We believe that prolonged complete starvation (for 7 days) acts as a severe body "stress" whereas chronic malnutrition does not act as a stress, although the "soil" in malnourished animal's tissue is depleted or changed sufficiently to decrease incidence of formation of spontaneous tumors.

Our findings tend to support those of Buinauskas *et al.*(3) revealing increased susceptibility of animals having the stress of an operation (celiotomy) to inoculated Walker 256 cells.

Summary. The effect of acute starvation and dehydration in rats, on "takes" of Walker 256 cells following their injection subcutaneously has been investigated. Our results indicate that starvation and dehydration for 48 hours do not increase susceptibility of the rat to subcutaneous inoculation of Walker tumor, but if starvation is continued for 7 days there is increase in percentage "takes" of the tumor as compared to incidence in normal healthy animals. There is also an increase in "takes" of implanted tumor in young rats 28 days of age compared to more mature rats.

1. Tannenbaum, A., Silverstone H., *Cancer Res.*, 1949, v9, 724; 1950, v10, 577.

2. Talalay, P., Takano, G. M., Huggins, C., *ibid.*, 1950, v12, 834.

3. Buinauskas, P., McDonald, G. O., Cole, W. H., *Ann. Surg.*, 1958, v148, 642.

Received April 20, 1960. P.S.E.B.M., 1960, v104.

Effects of Respiratory Inhibitors on Glucose and Protein Utilization and Growth in Strain L Cells.* (25876)

D. M. PACE AND L. M. ELROD†

Dept. of Physiology, University of Nebraska, Lincoln

Azide, urethan, arsenite and fluoride are well known respiratory inhibitors and have been used as such in innumerable investigations. Except for the work of Cailleau *et al.* (2) on azide, there have been no reports on effects of these substances on strain L cells. Westfall *et al.*(14) studied glucose utilization by strain L cells and although amino acid requirements have been ascertained to some extent, no measurements of total protein utilization by these cells have been reported. In the following report, results of experiments made upon strain L cells by exposing them to these several substances and noting the effects on glucose and protein utilization, as well as upon cell numbers and cell division, are recorded.

Materials and methods. Earle's medium, consisting of 40% horse serum, 40% balanced salt solution and 20% (1:1) chicken embryo extract was used. This medium was modified for control cultures by using 39% Earle's balanced salt solution and 1% triple distilled water. The test medium also contained 39% balanced salt solution and 1% of desired concentration of inhibitor solution. For these experiments, 1 ml of cell suspension containing approximately 1 million cells was placed in each of 20 Carrel D-3.5 flasks. The medium was removed 48 hours later and in each of 10 flasks, was replaced with 2 ml of fresh

* This investigation supported in part by research grants from Nat. Science Fn. and U.S.P.H.S.

† Present address: Dept. of Biology, Westminster College, Fulton, Mo.

TABLE I. Effect of Sodium Fluoride, Sodium Azide, Sodium Arsenite and Urethan on Growth in Earle's Strain L Cells. Initial cell count, approximately 1 million cells/ml. Cell numbers are avg obtained for 20 cultures.

Molar conc. of inhibitor		Cell numbers 10^6/ml Control	Test	Growth (% of control)
Fluoride	10^{-2}	2.57	.00	00
	10^{-3}	3.07	2.33*	76
	10^{-4}	1.90	2.06	108
	10^{-5}	3.45	3.60	104
Azide	10^{-3}	3.38	.00	00
	10^{-4}	2.99	1.09*	37
	10^{-5}	3.25	3.21	99
Arsenite	10^{-4}	3.62	.00	00
	10^{-5}	3.09	.58*	19
	10^{-6}	3.37	3.25	96
	10^{-7}	3.45	3.45	100
Urethan	10^{-1}	3.67	.00	00
	10^{-2}	2.78	2.24	81
	10^{-3}	3.51	3.50	100
	10^{-4}	3.43	3.53	103

* Highly significant (P equals less than 1%).

medium containing the inhibitor. The 10 control flasks also received 2 ml of medium but without inhibitor. Glucose and protein determinations were made 72 hours later as follows: The medium was removed from 5 test flasks and 5 control flasks and cells separated by centrifugation. Glucose remaining in medium of each flask was determined colorimetrically by means of Somogyi(12) method. Duplicate measurements were made using "Glucostat"‡ enzymatic reagent. Total unused protein was ascertained by Biuret reaction (Cornall *et al.*,(3)). These results were checked by using Tsuchiya's reagent and measuring precipated protein (Simmons and Gentzkow(10)). At the same time, mitotic counts and nuclei counts were made; the latter by nuclei enumeration method of Sanford, Earle *et al.*(9).

Results. Counts were made 72 hours after exposure of cells to inhibitors (120 hours after cultures had been started). The results are presented in Table I. Cells in control cultures averaged 3.17 million/ml after 5 days. Sodium fluoride (10^{-2} M), sodium azide (10^{-3} M), sodium arsenite (10^{-4} M) and urethan (10^{-1} M), killed the cells. Slightly lower concentrations retarded proliferation, although 10^{-2} M urethan appeared to have no significant effect. Statistical analyses were made by the method of group comparisons of Snedecor(11).

Results of studies on utilization of glucose by strain L cells are presented in Table II. Glucose in control medium was in most cases completely depleted. There is 28% and 23% reduction in glucose uptake in 10^{-2} M sodium fluoride and 10^{-4}M sodium arsenite, respectively, while urethan and azide apparently exert little if any effect in concentrations used.

The effect of inhibitors on protein utilization is shown in Table III. Sodium azide (10^{-3} M) and sodium fluoride (10^{-3} M) inhibit protein uptake by 92 and 39%, respectively. Concentrations of 10^{-5} M and 10^{-6} M arsenite increase protein uptake by 87 and 110%, respectively. A concentration of 10^{-2} M urethan increases protein uptake by 39%. An unusual effect, for which we have no explanation, was noted in 10^{-5} M azide, where protein content of the medium increased by 93%.

Examination of cultures in which cells had been stained, revealed a mitotic index of 10.5% for untreated (control) cells. It was much lower for all experimental cultures ex-

TABLE II. Effect of Sodium Fluoride, Sodium Azide, Sodium Arsenite and Urethan on Glucose Utilization in Earle's Strain L Cells. Initial glucose concentration, 1 mg/ml. Each figure for glucose uptake is an avg obtained for 10 cultures.

Molar conc. of inhibitor		Glucose utilization, mg/ml Control	Test	% of control
Fluoride	10^{-2}	1.	.72†	72
	10^{-3}	1.	.85†	85
	10^{-4}	1.	.93†	93
	10^{-5}	1.	1.	100
Azide	10^{-3}	.91	1.	110
	10^{-4}	1.	1.	100
	10^{-5}	1.	1.	100
Arsenite	10^{-4}	1.	.77†	77
	10^{-5}	1.	.77†	77
	10^{-6}	1.	1.	100
	10^{-7}	.99	1.	101
Urethan	10^{-1}	1.	.97*	97
	10^{-2}	1.	1.	100
	10^{-3}	1.	.92†	92
	10^{-4}	1.	.99	99

* Significant difference (P equals less than 5%).
† Highly significant difference (P equals less than 1%).

‡ "Glucostat" reagent was obtained from Worthington Biochemical Corp., Freehold, N. J.

TABLE III. Effect of Sodium Fluoride, Sodium Azide, Sodium Arsenite and Urethan on Protein Utilization in Earle's Strain L Cells. Avg initial protein concentration, 29 mg/ml. Each figure for protein utilization is an avg obtained for 10 cultures.

	Molar conc. of inhibitor	Protein utilization, mg/ml Control	Test	% of control
Fluoride	10^{-2}	3.6	3.1	86
	10^{-3}	8.6	5.2*	61
	10^{-4}	5.0	4.7	94
	10^{-5}	3.3	3.9	118
Azide	10^{-3}	1.3	.1	8
	10^{-4}	5.6	7.5	134
	10^{-5}	1.5	+1.4	−93
Arsenite	10^{-4}	3.1	4.8	155
	10^{-5}	4.6	8.6*	187
	10^{-6}	3.1	6.5	210
	10^{-7}	3.5	4.0	114
Urethan	10^{-1}	6.6	8.5	129
	10^{-2}	4.9	6.8*	139
	10^{-3}	1.9	3.1	163
	10^{-4}	3.9	5.0	128

* Significant difference (P equals less than 5%).

cept azide (10^{-4} M), and zero for those concentrations that inhibited growth.

The cells were also examined to ascertain number of nuclei in each. In control cultures 4% of cells were multinucleate. When exposed to 10^{-4} M azide there was a 17% increase in multinucleate cells. Cultures exposed to urethan (10^{-2} M) and fluoride (10^{-3} M) possessed very few multinucleate cells—2%.

Giant cells were frequently observed in both control and test cultures. They were of 2 types, those having a single very large nucleus and those with several smaller nuclei. In cells exposed to 10^{-2} M fluoride no giant single nucleate cells were found. The greatest number of giant cells was noted in azide (10^{-4} M). In large multinucleate cells there were many with variable numbers of nuclei and many with nuclei of different sizes, but no mitotic figures were seen.

Discussion. Our results indicate that 10^{-4} M azide has little effect on glucose utilization, but inhibits cell growth by 63% which conforms with findings of Cailleau *et al.*(2) that azide (5×10^{-4} M) inhibits growth in strain L cells by 89%. Of 4 inhibitors used, arsenite (10^{-5} M) exerts the greatest inhibition, 81%; fluoride (10^{-3} M) decreases growth by 24%. Urethan exerts no significant inhibition.

The failure of azide to affect glucose utilization could be due to its "uncoupling" action allowing oxidation but inhibiting oxidative phosphorylation (Loomis and Lipmann(5); Mehler(6)). Another possible explanation might be in the findings of Stannard(13), that azide acts on one of 2 parallel respiratory systems.

Only preliminary studies have been made on protein utilization of tissue cells (Pasieka *et al.*,(8)). Results of our study show increase in protein utilization when arsenite or urethan is present in certain concentrations; fluoride and azide have an inhibiting effect. Merchant and Kahn(7) found that suspension cultures of strain L cells produce a collagen-like fibrous protein which is released into the medium. Although the work presented here was done differently than that of Merchant and Kahn, we did find considerable variation in protein utilization in control cultures (8.6 to 1.3 mg/ml). This might be explained by assuming that the cells at times utilize protein from the medium and at other times release soluble proteins into the medium, but in stationary cultures the protein remains in solution and does not coalesce into fibers.

In this investigation the mitotic index was lowered by all inhibitors except azide. Bullough(1) reported mitotic inhibition in azide and claimed this was due to interference with carbohydrate metabolism. Under the conditions of our experiments, azide did not appear to interfere with carbohydrate metabolism or lower the mitotic index.

Lambert(4) and many others reported that multinucleate giant cells were formed by fusion of many uninucleate cells and that a glass substrate might act as a stimulus for fusion. Our observations indicate that mode of formation may be by division of the nucleus without cytoplasmic cleavage, or in many instances cytoplasmic division occurs followed immediately by fusion of the cells.

Summary. 1) Effects of sodium fluoride, sodium azide, sodium arsenite and urethan on growth, glucose utilization, protein utilization and mitosis in strain L cells were studied. 2)

Cell populations in control cultures averaged 3.17 million/ml after 5 days; had utilized an average of 0.99 mg of glucose and 4.0 mg of protein/ml of medium. 3) Fluoride (10^{-3} M), azide (10^{-4} M) and arsenite (10^{-5} M) decreased cell numbers by 24, 63 and 81%, respectively. Although urethan (10^{-1} M) killed the cells, a concentration of 10^{-2} M had little effect. 4) Fluoride (10^{-2} M), arsenite (10^{-4} M) and urethan (10^{-3} M) inhibited glucose uptake by 28, 23 and 8%, respectively. Azide (10^{-3} M) produced 10% acceleration of glucose uptake. 5) Azide (10^{-3} M) and fluoride (10^{-3} M) inhibited protein uptake by 92 and 39%, respectively. Arsenite and urethan accelerated protein uptake in all concentrations. 6) The mitotic index of untreated cells was 10.5. All inhibitors except azide produced a lowered index. 7) In control cultures, 4% of cells were multinucleate. Azide (10^{-4} M) increased these forms to 17%.

1. Bullough, W. S., *Biol. Rev.*, 1952, v27, 133.

2. Cailleau, R., Moss, S., Siegel, B. V., *J. Nat. Cancer Inst.*, 1956, v16, 1011.

3. Cornall, A. G., Bardawill, G. J., David, M. M., *J. Biol. Chem.*, 1949, v177, 751.

4. Lambert, R. A., *J. Exp. Med.*, 1912, v15, 510.

5. Loomis, W. F., Lipmann, F., *J. Biol. Chem.*, 1948, v173, 807.

6. Mehler, A. H., *Introduction to Enzymology.* Acad. Press, N. Y., 1957, p385.

7. Merchant, D. J., Kahn, R. H., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 359.

8. Pasieka, A. E., Morton, H. J., Mcrgan, J. F., *J. Nat. Cancer Inst.*, 1956, v16, 995.

9. Sanford, K. K., Earle, W. R., Evans, V. J., Waltz, H. K., Shannon, J. E., Jr., *ibid.*, 1951, v11, 773.

10. Simmons, J. S., Gentzkow, C. J., *Laboratory Methods of U. S. Army.* 1944. Lea and Febiger, Philadelphia. 5th ed. p172.

11. Snedecor, G. W., *Statistical Methods.* Iowa State Coll. Press, Ames, 1948, 4th ed. p75.

12. Somogyi, M., *J. Biol. Chem.*, 1945, v160, 69.

13. Stannard, J. N., *Am. J. Physiol.*, 1939, v126, 196.

14. Westfall, B. B., Evans, V. J., Shannon, J. E., Jr., Earle, W. R., *J. Nat. Cancer Inst.*, 1953, v14, 655.

Received April 21, 1960. P.S.E.B.M., 1960, v104.

Extraction and Concentration of Mammogenic Fractions from Anterior Pituitary Gland.* (25877)

HENRY C. DAMM† AND C. W. TURNER
Dept. of Dairy Husbandry, University of Missouri, Columbia

The concept of homogeneity of lactogenic and mammogenic (mammary gland growth promoting) hormones has been disputed for many years. Older studies(1) showing wide differences in relative amount of these hormones have been repeated using improved assay technics for both hormones(2). Since recent evidence(3) indicates that lactogenic hormone discharge from anterior pituitary gland (AP) does not occur until late pregnancy, or until after over 50% of mammary gland growth has taken place, it would appear incongruous with physiological facts to accept the idea that lactogenic hormone is pituitary agent primarily responsible for mammary gland growth during pregnancy. Critics of the mammogen concept have suggested need of extracting a preparation from the pituitary rich in mammogen and free of lactogenic hormone. The present report indicates our progress. It will be shown that mammogenic hormone is poorly extracted by solvents which effectively remove other established hormones (4). Evidence will be presented indicating that after other hormones are removed from AP, a mammogenic factor may be extracted and concentrated from the residue.

Materials and methods. To remove gona-

* Contribution from Mo. Agr. Exp. Sta. Journal Series No. 1912. Approved by the Director. This investigation was supported by grants from U.S.P.H.S. and Am. Cancer Soc.

† Public Health Service Research Fellow of Nat. Cancer Inst.

dotropins, ACTH, TSH, growth and lactogenic hormone, acetone dried AP powder was thoroughly mixed with 16 volumes of 56% ethanol and 4% butanol. The suspension was brought to pH 12.5 by addition of 1 N NaOH, then titrated back to pH 10.0 with 1 N HCl. After stirring for 1 hour, residue was removed by bottle centrifuge. The residue was extracted 3 additional times, and each time pH of solvent was "over-shot" to 12.5, then adjusted to pH 10.5, 11.0, and 11.5 on second, third and fourth extractions, respectively. The residue was dried with acetone and ether and stored in a desiccator. Two mammogenic fractions were extracted from the residue, the first obtained by use of a sodium phosphate buffer solution and the second by one of isotonic saline. Phosphate buffer solution was composed of 0.6 g monosodium phosphate monohydrate and 2.2 g disodium phosphate anhydrous per liter distilled water. Isotonic saline was composed of 9 g C.P. sodium chloride per liter distilled water. A ratio of 1 part residue (by weight) to 40 parts solvent was used. Suspension was stirred at room temperature for 45 minutes, centrifuged, and clear supernatant saved. Residue was extracted in a similar manner 3 additional times and supernatants were pooled. In case of phosphate buffer system, pooled supernatants were brought to concentration of 56% ethanol and 4% butanol. During addition of 2 N NaOH, a precipitate formed at approximately pH 12.0. The precipitate was collected and dried with acetone. Buffer salts were removed by dissolving precipitate in water and dialyzing against distilled water which was agitated slowly for 8 hours. Four changes of water were required to remove phosphorous from solution. Precipitate was recovered on a Whatman No. 50 filter paper, dried with acetone and weighed. Yield was approximately 0.12% of initial residue, or 0.08% of starting acetone dried AP powder. This fraction was an amorphous, grey powder (fraction A). Upon assay(5), this fraction contained less than 0.03% lactogen, $p = .99$. For the saline solution, all extracts were pooled and saturated with sodium chloride, then chilled in deep freeze. Precipitate was collected on Whatman No. 50 filter paper, dried with acetone, and weighed. Yield was approximately 0.10% of the initial residue, or about 0.07% of the starting acetone dried AP powder. This fraction was more crystalline and white (fraction B). This fraction contained no trace of lactogen. To produce a suitable standard mammary gland growth curve for comparison of mammogenic potencies of a highly purified lactogenic preparation(6) and mammogenic fraction A, 0.75 μg estradiol benzoate (EB) plus varying amounts of progesterone were injected/day for 10 days to male mice previously primed with diethylstilbestrol. Graded amounts of lactogenic hormone plus 0.75 μg EB/day and graded amounts of fraction A plus 0.75 μg EB/day were administered to groups of estrogen primed male mice for 10 days. Mammary gland growth determined by amount of desoxyribonucleic acid was compared, and relative potencies of the 2 protein fractions were calculated (Table I). In second experiment, female mice were ovariectomized, and groups injected with estrogen and estrogen plus progesterone for 19 days, to approximate gestation period in mouse. To determine relative

TABLE I. Lobule-Alveolar Growth Stimulated by Progesterone, Mammogen and Lactogen in the Male Mouse.

Substance	Total 10-day dose (mg)	No. of animals	Total DNA mean (mg)
Progesterone*	10.0	9	3.25
	5.0	7	2.96
	1.25	8	2.68
Lactogen (Cole & Li)*	4.0	8	3.65
	2.0	9	2.80
	1.0	10	2.16
	.5	5	2.10
Conc. mammogen* (fraction A)	.1	10	3.44
	.05	8	3.07
	.025	8	3.05
	.0125	9	2.82

* Plus .75 μg estradiol benzoate/day.

SUMMARY

Summary	a	b	Syx	Relative potency
Progesterone	2.60	.612	.078	1.0
Lactogen (Cole & Li)	1.71	1.76	.286	1.59 .89–2.87
Conc. mammogen	2.75	.631	.140	223.3 96.6–515.5

TABLE II. Effects of Anterior Pituitary Fractions on Mammary Gland Growth in Ovariectomized Female Mice.

Treatment, daily dose	No. of animals	DNA/mg DFFT, μg	DFFT, mg	Total DNA, mg	Body wt mean, g	Total DNA per 10 g body wt, mg
Controls, ovariectomized	15	27.8 ± 5.8	64.8	1.805 ± .387	25.6	.706 ± .151
1.0 μg EB*	11	26.6 ± 3.9	102.3	2.720 ± .406	30.1	.902 ± .135
Idem + 3 mg progesterone	12	26.4 ± 3.3	170.6	4.528 ± .564	34.6	1.304 ± .163
" + 4 μg Ant. pituitary	10	38.4 ± 5.3	171.3	6.578 ± .912	35.5	1.853 ± .257
" + " initial residue	9	36.0 ± 5.4	137.4	4.941 ± .737	34.1	1.451 ± .216
" + .01 mg Fraction B	9	38.4 ± 4.7	127.9	4.912 ± .607	35.1	1.403 ± .173
" + " " A	11	31.6 ± 5.7	112.7	3.564 ± .672	32.6	1.162 ± .206
18-day pregnant†	15	57.0 ± 1.7	125.6	7.007 ± .281	38.5	1.832 ± .021

* Estradiol benzoate.

† Brookreson and Turner(8).

mammogenic potency of AP powder, initial residue and mammogenic fractions A and B, equivalent amounts of these fractions were injected along with .75 μg estradiol benzoate into groups of ovariectomized female mice for period of 19 days. Mammary glands were removed and growth determined by DNA measurement (Table II).

Results. Estrogen and progesterone have been shown to promote extensive lobule-alveolar mammary gland growth in intact albino mice(7). Good growth as determined by DNA and whole mount technics was produced by 1 mg progesterone plus 0.75 μg estradiol benzoate per day for 10 days (Table I). The highly purified lactogenic preparation produced approximately comparable results when 0.4 mg was injected per day with estrogen. Calculated relative potency was 1.59, indicating that this preparation is slightly more potent than progesterone. Mammogenic fraction A produced good development when 0.01 mg was injected with estrogen daily for 10 days. The mean relative potency was 223.3, indicating much greater mammary gland stimulating potency than either highly purified lactogen or progesterone.

Results of 19-day injection of single doses of various fractions into ovariectomized female mice (Table II) show that estrogen plus crude AP powder produced growth comparable to that noticed in the 18-day pregnant primiparous mouse(8), which was notably better than growth produced by estrogen and progesterone, initial residue or either of mammogenic fractions.

While it required 4.0 mg initial residue to produce mammary gland growth equivalent to that produced by 3.0 mg progesterone, only 0.01 mg fraction A or fraction B were required to produce equivalent amounts of growth.

Discussion. Methods have been described for extraction and concentration of fractions from the anterior pituitary gland which, in intact male albino mice or ovariectomized female mice, exhibits marked mammogenic properties. That crude AP powder produced better growth than progesterone, initial residue or either of the mammogenic fractions could be explained on the basis that other hormones injected tended to equalize amount of circulating AP hormones in individual animals, thus permitting mammary glands to reach maximum development under stimulation by mammogenic hormone.

Existence of a separate specific mammogenic hormone has been doubted due to the presence of mammogenic activity in lactogenic extracts of highest potency. However, the fact that 0.01 mg of mammogenic extract produced lobule-alveolar growth approximately equal to that produced by 0.4 mg of lactogen indicates a maximum contamination of the latter with 0.71% of mammogen. This would indicate that mammogen is extracted to a slight extent by methods of lactogen preparation.

Summary. It has been shown that appreciable quantities of mammogenic factor are left in initial residue of anterior pituitary gland after extraction of the known hormones by usual technics. This factor was extractable by either a monosodium-disodium phos-

phate buffer solution or a saline solution. The active factor precipitated from the phosphate buffer system along with phosphate salts after addition of ethanol and butanol and adjusting the pH to 12.5. Phosphate salts were subsequently removed by dialysis, during which mammogenic hormone precipitated out of solution. Active factor was precipitated from saline solution by saturation of solution with NaCl and chilling. Resulting light grey, amorphous powder obtained by phosphate extraction was 223 times more potent in promoting mammary gland growth than progesterone, and contained negligible, if any, lactogenic activity. A highly purified lactogenic preparation assayed for mammogenic activity at the same time was only 1.59 times as potent as progesterone. On this basis the lactogen need contain no more than 0.71% of mammogen to produce the biological response observed.

1. Mixner, J. P., Turner, C. W., *Mo. Agr. Exp. Sta. Res. Bull.*, 1943, #378.

2. Damm, H. C., Turner, C. W., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 471.

3. Grosvenor, C. E., Turner, C. W., *Endocrinology*, 1960, v66, 96.

4. Bergman, A. J., Turner, C. W., *Mo. Agr. Exp. Sta. Res. Bull.*, 1942, #356.

5. Grosvenor, C. E., Turner, C. W., *Endocrinology*, 1958, v63, 530.

6. Cole, R. D., Li, C. H., *J. Biol. Chem.*, 1955, v213, 197.

7. Damm, H. C., Turner, C. W., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 466.

8. Brookreson, A. D., Turner, C. W., *ibid.*, 1959, v102, 744.

Received April 25, 1960. P.S.E.B.M., 1960, v104.

Effect of Corticotropin and Growth Hormone on Allantoin Synthesis and Excretion in the Dog.* (25878)

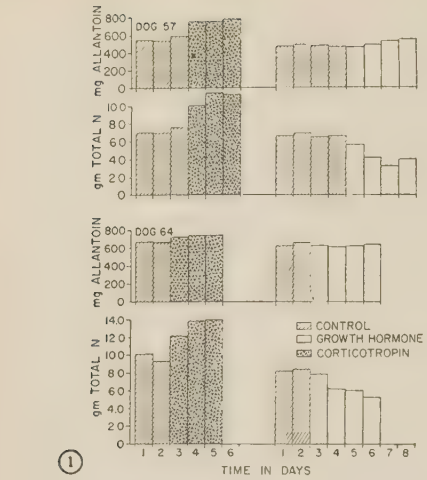
H. C. CHOITZ AND O. H. GAEBLER

Edsel B. Ford Inst. for Medical Research, Henry Ford Hospital, Detroit, Mich.

The effect of corticotropin on excretion of end products of purine metabolism seems to vary with species. In dogs urinary output of allantoin is increased by massive doses of corticotropin, presumably as a result of accelerated excretion as well as increased allantoin synthesis(1). On the other hand, corticotropin apparently does not increase allantoin synthesis in the rat, and the renal tubule has been suggested as a possible site of action of hormone on allantoin excretion(2). Likewise uricosuria in human subjects after administration of corticotropin has been attributed to increased renal clearance of uric acid rather than increased synthesis(3). A recent investigation of effects of corticotropin and growth hormone on utilization of N^{15} from individual sources(4) afforded an opportunity to secure data on incorporation of the isotope, ingested as labeled glycine or ammonium citrate, into urinary allantoin of normal dogs, during control experiments, and after administration of corticotropin or growth hormone. Experiments with growth hormone seemed relevant because of its importance in nitrogen metabolism in general, and in view of reports of its positive action on mitotic activity and synthesis of nucleic acids(5,6).

Materials and methods. Two adult mongrel bitches were used as experimental animals. Details regarding care, diet, source of hormones, and preparation of labeled compounds have been described(4). Corticotropin was given subcutaneously in 2 doses of 10 units each (9:00 a.m. and 3:00 p.m.) for 3 successive days. Growth hormone, in doses of 5 mg dissolved in 1 ml of normal saline, was administered subcutaneously on 4 consecutive days. The labeled compound was fed with the morning ration on second day of corticotropin treatment and on third day of growth

* This study was supported, in part, by Research Grant from Nat. Inst. of Arthritis and Metabolic Dis., N.I.H.



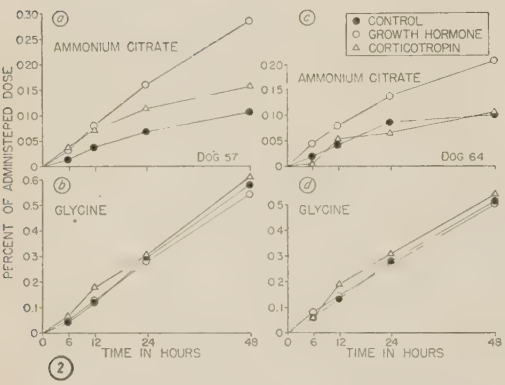


FIG. 1. Effect of corticotropin and growth hormone on excretion of total nitrogen and allantoin.

FIG. 2. Incorporation of N^{15} from ammonium citrate and glycine into urinary allantoin, expressed as cumulative % of administered dose: *a, b,* Dog 57; *c, d,* Dog 64.

hormone therapy. Urine was collected under toluene at 6, 12, 24 and 48 hour intervals after tracer had been fed. Each collection period was terminated by catheterizing and washing the bladder. Nitrogen of allantoin was isolated as the dixanthylurea derivative in a manner similar to that of Allen and Cerecedo (7). In this method, preexisting urea is first decomposed with urease. Allantoin is then successively hydrolyzed with base to allantoic acid, and finally with acid to urea and glyoxylic acid. Addition of xanthydrol to the acid solution precipitates urea formed. To a sample of urine, which would yield at least 0.5 mg of allantoin nitrogen, were added 5 ml of phosphate buffer, pH 7.0, and 4 ml of urease solution, after which incubation was carried out at 40°C for 2 hours. The sample was then made alkaline to about pH 12 with 50% sodium hydroxide, and heated on steam cone for 20 minutes. After cooling, sample was acidified to about pH 2 with concentrated hydrochloric acid, treated with decolorizing carbon, heated on steam cone for 10 minutes, and filtered hot. The filtrate was treated with enough xanthydrol dissolved in methanol-glacial acetic acid (80:20) to precipitate the urea. The precipitate was collected by centrifugation, washed successively with water, alcohol, and ether, and dried at 110°C. Procedures for digesting the compound, distilling the ammonia obtained, and analyzing its nitrogen in the mass spectrometer were the same as in preceding study(4). In several preliminary trials, acidification of incubation mixture and addition of xanthydrol gave no alcohol-insoluble precipitate, indicating that decomposition of urea originally present as such was complete. Recovery of allantoin nitrogen was not quantitative, but averaged about 60% of that found colorimetrically by the method of Young and Conway(8).

Results. Corticotropin increased urinary excretion of allantoin, while growth hormone had little or no effect (Fig. 1). The increase was much more marked in Dog 57 than in Dog 64. A minor part of this difference may have been due to the fact that Dog 57 weighed about 3 kilos less than Dog 64, while both received the same dosage of corticotropin. In all experiments treatment with corticotropin or growth hormone produced the expected decrease or increase in total urinary nitrogen.

Amounts of N^{15} incorporated into urinary allantoin during 48 hours after feeding labeled compounds, and specific enrichments (μg N^{15}/g allantoin N) are given in Table I. Values for specific enrichment represent average of entire period. The nitrogen of glycine was utilized to considerably greater extent than that of ammonium citrate for synthesis of allantoin, since almost identical amounts of isotope were fed in each case.

Corticotropin did not significantly alter specific enrichment of allantoin nitrogen. A possible interpretation of this result is that in-

TABLE I. Incorporation of N^{15} into Urinary Allantoin in 48 Hours.

Dog	Source* of N^{15}	Treatment	N^{15} incorporated μg	$\mu g/g$ allantoin N
57	Amm. cit.	None	23	62
"		Growth hormone	61	139
"		Corticotropin	34	63
64		None	21	43
"		Growth hormone	44	103
"		Corticotropin	23	45
57	Glycine	None	126	316
"		Growth hormone	117	307
"		Corticotropin	133	289
64		None	111	221
"		Growth hormone	109	223
"		Corticotropin	118	224

* Ammonium citrate-N^{15} contained 21.46 mg N^{15}, and glycine-N^{15} contained 21.65 mg N^{15}.

creased excretion reflected increased synthesis of allantoin, but that the proportion of unlabeled and labeled precursors involved in this process remained the same as in control experiments.

Excretion of N^{15} in allantoin nitrogen, expressed as cumulative per cent of administered dose of N^{15}, is illustrated in Fig. 2. Similar results were obtained with both dogs. There was a relatively large increase in utilization of ammonium citrate-N^{15} for allantoin synthesis when growth hormone was given. Since no comparable increase occurred in the case of glycine-N^{15}, this may be a reflection of hormone's effect, or lack of effect, on pool size of different allantoin precursors.

It has been suggested that there are in the human 2 pathways for incorporation of labeled precursors into urinary uric acid(9,10). One pathway presumably proceeds *via* nucleotide intermediates, which are oxidized to uric acid before they can be incorporated into nucleic acids. In the second pathway, the labeled precursor finds its way into cell nucleic acids. The labeled purines are then oxidized to uric acid as cells break down. Any uric acid arising from first pathway would be labeled more rapidly than that formed from breakdown of nucleic acids, since turnover rate of the latter is slow.

It cannot be definitely concluded from our data that a similar situation exists in the dog. However, prompt appearance of ingested N^{15} in urinary allantoin might suggest nucleotides, rather than nucleic acids, as principal precursors during short interval studied.

Summary. 1. Growth hormone increased amount of ammonium citrate-N^{15} utilized for allantoin synthesis in dogs. Corticotropin did not have this effect. Neither hormone increased incorporation of N^{15} from glycine into allantoin. 2. Corticotropin increased output of urinary allantoin in dogs, without markedly reducing the specific enrichment with N^{15} in this excretory product. One possible interpretation of this finding is increased synthesis of allantoin, without qualitative changes in its origin.

1. Fajans, S. S., Conn. J. W., Johnson, D. V., Christman, A. A., in *Proc. of 2nd Clinical ACTH Conf.,* The Blakiston Co., N. Y., 1951, vI, 290.

2. Friedman, M., Byers, S. O., *Am. J. Physiol.,* 1950, v163, 684.

3. Benedict, J. D., Forsham, P. H., Roche, M., Soloway, S., Stetten, D., Jr., *J. Clin. Invest.,* 1950, v29, 1104.

4. Gaebler, O. H., Glovinsky, R., Lees, H., Kurrie, D., Choitz, H. C., *Endocrinology,* 1959, v65, 283.

5. Liu, K. H., Winnick, T., *Exp. Cell Research,* 1954, v7, 238.

6. Leblond, C. P., Carriere, R., *Endocrinology,* 1955, v56, 261.

7. Allen, F. W., Cerecedo, L. R., *J. Biol. Chem.,* 1931, v93, 293.

8. Young, E. G., Conway, C. F., *ibid.,* 1942, v142, 839.

9. Benedict, J. D., Roche, M., Yü, T. F., Bien, E. J., Gutman, A. B., Stetten, D., Jr., *Metabolism,* 1952, v1, 3.

10. Wyngaarden, J. B., Seegmiller, J. E., Laster, L., Blair, A., *ibid.,* 1959, v8, 455.

Received April 28, 1960. P.S.E.B.M., 1960, v104.

Effects of Dextran on Cortisone-Induced Hyperlipemia in Rabbits.* (25879)

Marcus A. Rothschild, Murray Oratz, Ernest Wimer and Sidney S. Schreiber
(Introduced by Milton Levy)
Vet. Admin. Hospital; and Dept. of Medicine, N. Y. University School of Medicine.

Following administration of cortisone, rabbits commonly develop marked hyperlipemia (1,2), associated primarily with increase in plasma triglycerides(2). However, as an incidental observation we noted that hyperlipemia failed to develop in cortisone-treated rabbits maintained on high doses of dextran given intravenously prior to and concurrently with cortisone administration. Further investigation is the subject of the present report.

Methods. Female rabbits weighing from 3-5 kg were kept in metabolism cages and fed standard rabbit ration *ad lib.* Dextran (Laros Lot H. average molecular weight of 188,000) was prepared as 6% solution in saline and sterilized by passage through bacterial filter. Daily doses of 1.0-1.5 g of dextran were administered intravenously and cortisone acetate (3 mg/kg body weight/day) was administered subcutaneously. All animals on cortisone therapy received 75,000 units of procaine penicillin and 0.25 g of streptomycin intramuscularly every 3rd day as well as 5 meq of potassium chloride in drinking water. Blood samples were obtained after fasting 12-16 hours. Intravenous glucose tolerance tests (400 mg of glucose/kg body weight) were performed in 12 control rabbits, 12 rabbits receiving cortisone 2-3 weeks, 4 rabbits receiving dextran 2 weeks and 3 rabbits receiving both dextran and cortisone 3 weeks. Intravenous glucagon† tolerance tests (2-3 μg glucagon/kg body weight) were performed in 7 rabbits as controls, in 4 rabbits after dextran and cortisone and in 3 after cortisone alone. Blood glucose was determined by Somogyi method(3). Total plasma lipids were determined gravimetrically(4). Plasma dextran levels were determined by method of Roe(5).

Results. Plasma lipid concentrations were determined in animals treated for various periods with cortisone or cortisone plus dextran with results shown in Fig. 1. When 5 normal rabbits were given dextran 10-15 days, their total plasma lipid was 104 $\pm$ 16 mg/100 ml compared to control levels in same animals of 116 $\pm$ 9 mg/100 ml. Differences between groups given cortisone alone and those given cortisone plus dextran at 10, 20 and 30 days (Fig. 1) are significant ($P<0.01$) for each period.

Six animals were started on cortisone treatment and subsequently given dextran while cortisone was continued for varying periods. There was no immediate effect on lipemia, but after 1 week a definite lowering of plasma lipids was obtained in 4 of 6 animals, numbers 10, 11, 3a, 5a (Fig. 2). Following withdrawal of dextran but continuance of cortisone treatment for another 10 days, rabbits 9, 10, and 11 showed increasing plasma lipid levels.

Intravenous glucose tolerance tests failed to reveal any significant differences between control and dextran groups or between cortisone and cortisone, dextran groups. Glucagon tolerance curves in 5 animals on cortisone therapy and 2 animals on cortisone-dextran therapy were not significantly different. Plasma dextran levels ranged between 1.3 and 2.2 g%. When added *in vitro,* dextran had no clearing effect on lipemic plasma.

Discussion. Non-sulfated mucopolysaccharides have been reported to effect a rapid reduction of elevated fat levels produced by intravenous injection of plasma from hyperlipemic patients into rats(6). Furthermore, administration of dextran to nephrotic rats (7) and to humans(8) has been associated with a decrease in plasma cholesterol and total lipids. It has been suggested that blockage of the reticuloendothelial system by dextran may have caused reduction in serum cholesterol(8). However, although accumulation of dextran has been demonstrated in re-

* Investigation supported in part by grant from U.S.P.H.S.

† Generously supplied by Dr. Otto K. Behrens, Eli Lilly & Co., Indianapolis.

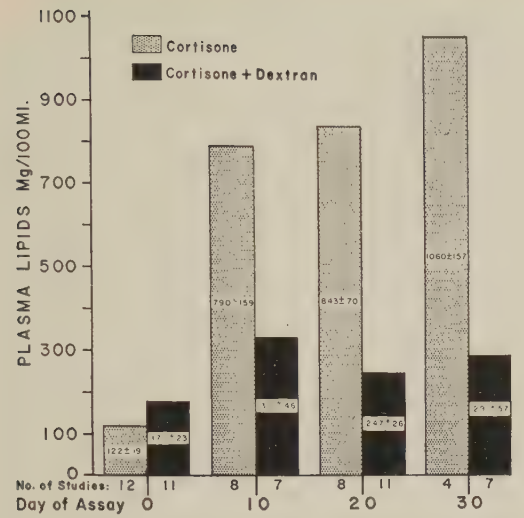


FIG. 1. Total plasma lipids (mg/100 ml ± S.E.) in rabbits on cortisone (3 mg/kg/day), and cortisone and dextran (1.0-1.5 g/day) after 0, 10, 20, and 30 days of therapy (see text).

ticuloendothelial cells(9), known reticuloendothelial blocking agents produce a rise in serum lipid levels(10) and a decreased clearance of cholesterol(11). In addition, dextran has been shown to increase rate of phagocytosis of carbon particles in the rat and mouse (12) and in the rabbit. Thus it is interesting to speculate that dextran effects its lipid clearing action by stimulation of the reticuloendothelial system. Cortisone administration on the other hand is associated with a depression of reticuloendothelial cell function(13) and dextran may have antagonized this action.

In addition to effects on fat metabolism, adrenal cortical hormones exert a significant influence on carbohydrate metabolism(14). However, our results failed to correlate inhibition of cortisone induced hyperlipemia with changes in glucose tolerance or glycogen stores.

Sulfated polysaccharides, such as dextran sulfate(15) and heparin(2) also exert an anti-hyperlipemic action and heparin increases clearance of cholesterol(11). The possibility exists that the lipemic clearing action of dextran may be due to conversion of dextran to a sulfated ester *in vivo*. However, it is doubtful that this is the basis for the clearing action of dextran since cortisone hyperlipemia is insensitive to heparin(16). Lipemic sera from cortisone-treated animals appears to contain some substance which inhibits the lipemic clearing factor(2).

In the present studies dextran not only inhibited development of lipemia but also

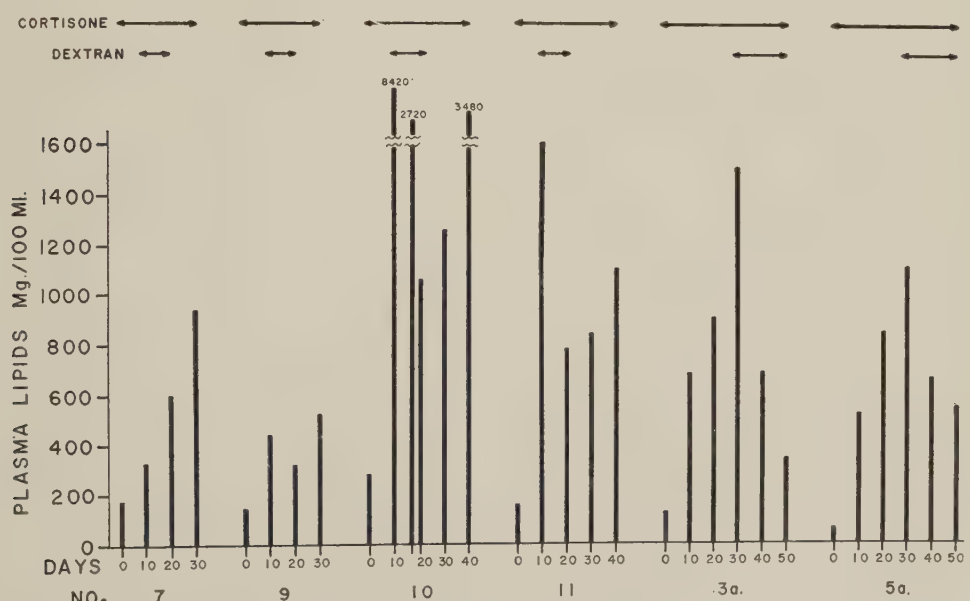


FIG. 2. Total plasma lipids (mg/100 ml) in 6 rabbits. Cortisone (3 mg/kg/day) was administered throughout the study in all the rabbits. Dextran (1.0-1.5 g/day) was administered from day 10 to 20 in rabbits 7, 9, 10, and 11, and from day 30 to 50 in 3a and 5a (see text).

partially reversed lipemia which had already appeared. This action could have resulted by blocking production of a clearing factor inhibitor or from stimulation of the lipid transporting system.

Summary. Dextran in doses of 1 to 1.5 g/day inhibited production of cortisone hyperlipemia in rabbits and partially reversed the hyperlipemia produced by cortisone. Alterations in fat transport, fat mobilization or in clearing factor action are considered as the basis for antagonism between dextran and hyperlipemic action of cortisone.

1. Rich, A., Cochran, T. H., McGoon, S. C., *Bull. Johns Hopkins Hosp.,* 1951, v88, 101.

2. Dury, A., *Ann. N. Y. Acad. Sci.,* 1959, v72, 870.

3. Somogyi, M., *J. Biol. Chem.,* 1952, v195, 19.

4. Natelson, S., *Microtechniques of Clin. Chem.,* Charles C. Thomas, Springfield, 1957, pp256.

5. Roe, J. H., *J. Biol. Chem.,* 1954, v208, 889.

6. Seifter, J., Baeder, R. H., Zarafonetis, C. I. D., Kalas, J., *Ann. N. Y. Acad. Sci.,* 1959, v72, 1031.

7. Heyman, W., Nash, G., Gilkey, C., Lewis, M., *J. Clin. Invest.,* 1959, v37, 809.

8. Morrison, A. B., *Quart. J. Exp. Physiol.,* 1951, v41, 51.

9. Perrson, B. H., *Nature,* 1952, v170, 716.

10. Feigenbaum, L., Byers, S. O., Freedman, M., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 530.

11. Neveu, T., Biozzi, G., Benacerraf, B., Stiffel, C., Halpern, B. N., *Am. J. Physiol.,* 1956, v187, 269.

12. Biozzi, G., Halpern, B. N., Benacerraf, B., Stiffel, C., Mouton, D., *Compt. Rend. des Seances de la Soc. de Biol.,* 1956, v150, 317.

13. Thomas, L., *Ann. N. Y. Acad. Sci.,* 1953, v56, 799.

14. Long, C. N. H., *Ciba Colloquia on Endocrinology,* Little, Brown and Co., Boston, 1953, v6, 136.

15. Cohen, H., Tudhope, G. R., *Brit. Med. J.,* 1956, v2, 1023.

16. Constantinides, P., Szasz, G., Dannach, M., 19th International Physiol. Cong. 1953, abs. 277.

Received April 28, 1960. P.S.E.B.M., 1960, v104.

Some Pharmacological Actions of Amphenidone* A New Psychotherapeutic Drug. (25880)

JOHN F. REINHARD, OSVALDS J. PLEKSS AND JOHN V. SCUDI†
Research Dept., Wallace & Tiernan, Belleville, N. J.

A new chemical structure, 1-m-Aminophenyl-2-pyridone (amphenidone), is of interest in that it induces emotional stability without depression of psychic or motor functions(1). Because of promise shown in clinical trials, some of its pharmacological actions are described below.

Methods. Potentiation of a subhypnotic dose of hexobarbital (50 mg/kg) by amphenidone and other drugs was measured as reported(2). Inhibition of the effects of LSD in the mouse (CF #1) was investigated using Haley's method(3). Anticonvulsant activity was determined in the mouse, using maximal electroshock (MES) and Metrazol (Met) seizures(4) and in the cat, in which clonic convulsions were evoked by injecting 50 mg/kg Metrazol, subcutaneously. Analgesic activity was assayed according to Eddy and Leimbach(5), using a 40-second exposure to the hot plate at 54-55°C. Cardiovascular and respiratory effects in the anesthetized (pentobarbital) dog were recorded kymographically using a Hg manometer and an Anderson inspirometer. Studies of isolated cat and rabbit hearts were conducted according to Langendorff(6). Effects on the nictitating membrane were studied using the method of Plummer *et al.*(7). Whenever linearity of dose-response relationships permitted, ED_{50}'s with 95% fiducial limits were computed graphically(8) and, in general, 10 or more animals were used at each of 3 or more doses.

Results. Loss of the righting reflex was noted only in response to toxic doses of the drug, *e.g.*, 500 mg/kg i.v. and 1000 mg/kg

* Amphenidone manufactured under trade-name Dornwal by Maltbie Labs., Wallace & Tiernan.

† With technical assistance of Domicele P. Skeivis, Richard B. Lawlor, and Bernice R. Strauss.

TABLE I. Hexobarbital Potentiation.

Compound	ED_{50} (95% limits), mg/kg p.o.	Mean sleeping time at ED_{50}, min.
Amphenidone	132 (106-165)	37
Hydroxyzine	250 (167-375)	51
Meprobamate	123 (108-140)	24
Phenaglycodol	62 (41- 94)	15

p.o. in the mouse, 400 mg/kg i.p. in the rat and 600 mg/kg i.v. in the dog. Doses within the toxic range for the cat (400 mg/kg p.o.) and dog (400 mg/kg i.v.) were not hypnotic.

When given together with subhypnotic dose of hexobarbital, amphenidone precipitated loss of the righting reflex presumably through central depression, although cord and vestibular mechanisms are not entirely excluded. Potent central depressants such as phenothiazines, reserpine, and phenobarbital characteristically gave low ED_{50}'s(2) in contrast with the large values listed for the first 3 compounds in Table I.

Amphenidone completely blocked the effects of LSD in the mouse. Unprotected animals exhibited Straub tail, tremors, aggressive behavior, and hyperexcitability(9). The oral ED_{50} of 110 (73-165) mg/kg provides a large margin of safety, the therapeutic index being 12. The rationale of this test involves the assumption that LSD or one of its congeners has a role in development of psychic disturbances. That this concept represents an oversimplification of complicated processes (10,11) is undoubtedly true. That it has a certain empirical value for the pharmacologist, however, has not been excluded(12).

Results summarized in Table II indicate anticonvulsant activity, particularly against Metrazol. In general, tranquilizers do not have this property; for example, the convulsant dose of Metrazol was lowered following reserpine(13), minimal electroshock seizure threshold was decreased significantly by chlorpromazine(14), and convulsions were actually precipitated in monkeys following chronic administration of chlorpromazine (15). Exploring the brain of unanesthetized monkeys with the aid of implanted electrodes, Delgado(16) found that amphenidone decreased spontaneous electrical activity of the cortex, septal areas, basal ganglia and thalamus. The decreased thalamic excitability suggests possible effectiveness against *grand mal* seizures.

Amphenidone appeared to exert an analgesic effect in the mouse. In the Eddy and Leimbach test the ED_{50}'s of amphenidone and codeine were 480 (445-520) and 145 (116-181) mg/kg p.o., respectively. At this high dose amphenidone may exert analgesic activity by suppression of motor function. Amphenidone did not reduce activity of morphine. In contrast to these findings, reserpine had no analgesic activity and actually reduced the effectiveness of morphine(17). Other studies, to be reported, indicate that perception of pain may be interrupted by amphenidone at cord levels. Peripheral sites of action appear to be excluded.

In the anesthetized dog no significant cardiovascular or respiratory effects were induced by intravenous doses of 25, 50, or 100 mg/kg of the drug. The ECG pattern (lead 2) was unchanged and actions of epinephrine, norepinephrine, acetylcholine, and serotonin on blood pressure were not affected. Electrically and chemically induced contractions of the nictitating membrane of the cat were not altered, even after large intravenous doses (200 mg/kg) of amphenidone. Perfusion of the isolated heart with massive doses (1000 μg) of the drug induced no changes in the coronaries or in amplitude or rate of ventricular contractions. In these experiments amphenidone was devoid of cardiovascular, respiratory, and autonomic activity.

Summary. Overt signs of CNS depression were elicited only in response to toxic doses of amphenidone. Selective central depressant activity was revealed by 1) blockade of

TABLE II. Anticonvulsant Activity.

Compound	ED_{50} (95% confidence limits), mg/kg p.o. MES*	Met†
Amphenidone	180 (165-190)	120 (97-149)
Paramethadione	175 (149-220)	110 (90-135)
Phenacemide	109 (98-122)	126 (107-149)
Diphenylhydantoin	11 (8- 16)	Nil
Trimethadione	Nil	470 (415-530)

* Maximal electroshock seizures.
† Metrazol convulsions.

stimulant effects of LSD; 2) anticonvulsant activity against electrically and chemically induced seizures; and 3) analgesic activity. In the anesthetized dog and cat, blood pressure and respiration were affected only by lethal doses of the drug. Large doses of amphenidone did not alter blood pressure responses to acetylcholine, epinephrine, norepinephrine or serotonin. Contractions of nictitating membrane in response to electrical or chemical stimulation were not altered by amphenidone. Normal functioning of isolated heart was not affected by addition of massive doses of the drug to perfusion fluid, nor, in the intact dog, was the ECG (lead 2) changed.

1. Landis, C., Whittier, J. R., Dillon, D., Link, R., *Am. J. Psychiatry,* 1960, v116, 747.

2. Reinhard, J. F., Scudi, J. V., PROC. SOC. EXP. BIOL. AND MED., 1959, v100, 381.

3. Haley, T. J., *Arch. int. Pharmacodyn.,* 1957, v110, 239.

4. Swinyard, E. A., Brown, W. C., Goodman, L. S., *J. Pharmacol. Exp. Therap.,* 1952, v106, 319.

5. Eddy, N. B., Leimbach, D., *ibid.,* 1953, v107, 385.

6. Langendorff, O., *Arch. ges. Physiol.,* 1895, v61, 291.

7. Plummer, J., Schneider, J. A., Barrett, W. E., *Arch. int. Pharmacodyn.,* 1954, v97, 1.

8. Litchfield, J. T., Jr., Wilcoxon, F., *J. Pharmacol. Exp. Therap.,* 1949, v96, 99.

9. Haley, T. J., *J. Am. Pharm. A.* (Sci. Ed.) 1957, v46, 428.

10. Koelle, G. B., *New Eng. J. Med.,* 1958, v25, 258.

11. Isbell, H., *Fed. Proc.,* 1956, v15, 442.

12. Fabing, H. D., *Neurology,* 1955, v5, 319.

13. Sacra, P., McColl, J. D., *Arch. int. Pharmacodyn.,* 1958, v117, 4.

14. Tedeschi, D. H., Benigni, J. P., Elder, C. J., Yeager, J. C., Flanigan, J. V., *J. Pharmacol. Exp. Therap.,* 1958, v123, 35.

15. Essig, C. F., Carter, W. W., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 726.

16. Delgado, J. M. R., Hoffman, H., Symmes, D., *Arch. int. Pharmacodyn.* April, 1960, v125, 161.

17. Sigg, E. B., Caprio, G., Schneider, J. A., *ibid.,* 1958, v97, 97.

Received May 3, 1960. P.S.E.B.M., 1960, v104.

Standardization of Electrocardiographic Recording in Repeated Experiments on Supine Anesthetized Dogs.* (25881)

C. NEWTON,† A. ELLIS AND S. ZAREMSKI (Introduced by L. N. Katz)

Cardiovascular Dept., Medical Research Institute, Michael Reese Hospital and Medical Center, Chicago, Ill.

There is great difficulty in obtaining reasonably constant electrocardiographic contour in repeated experiments upon dogs because the dog's heart is so mobile. This has been shown in this department(1) and also by Harris and Hussey(2) and Gross and Benison (3). The lateral position is no more successful as shown by Peterson, *et al.*(4), and the data of Lalich, *et al.*,(5). The former group(4) found better reproducibility in the supine position. This is the position we employed, taking not only limb leads but chest leads as well. Our study showed that the position employed minimized or eliminated the instability of serial records as seen in previous studies.

Materials and methods. A total of 32 electrocardiograms were taken on 12 healthy mongrel dogs weighing from 12 to 20 kg. One dog had 10 serial records and most had 3. The records were generally taken 3 days apart. The dogs were anesthetized with Pentothal Sodium, 20 mg/kilo, and placed supine on a V-shaped wooden table and tied down by means of lateral traction on the limbs. The electrode areas were shaved and coated with conducting jelly (Sanborn Redox). German silver plate electrodes were strapped on the 4 limbs just above the paws. Previous trials had shown that other limb positions or

* Aided by grant from Nat. Heart Inst.

† Postdoctorate Research Fellow, Nat. Heart Inst.

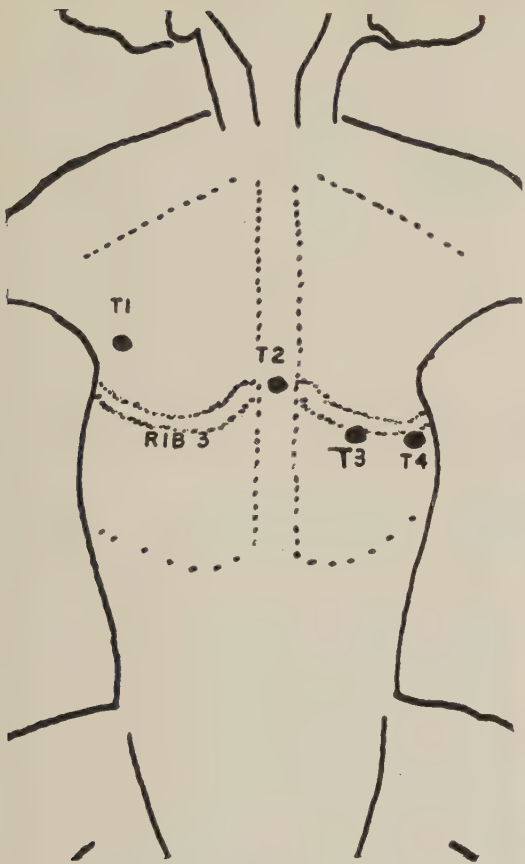


FIG. 1. Sketch of supine dog indicating position of sternum and third rib and location of chest positions for electrodes.

subcutaneous needle electrodes gave almost identical tracings. Suction electrodes, one inch in diameter, were used on the precordium. After preliminary testing of the various contour patterns over the precordium, 4 chest positions were selected (Fig. 1) because they had good anatomical landmarks and were representative of the chief contour types encountered. However, it is obvious that one type blended into another when the precordial electrode roamed over the chest. These precordial leads are not comparable to the human "V" leads in anatomical position or contour and therefore are labeled "T" leads. T_1 is in the 2nd right intercostal space at anterior axillary line. T_2, 3rd rib at midsternum. T_3, 4th left intercostal space midway between sternum and left anterior axillary line. T_4, 4th left intercostal space at anterior axillary line.

Results. Although there were variations in electrocardiographic contour between dogs, each dog's electrocardiogram was characteristic and essentially constant in successive tracings, (Fig. 2). This consistency was seen in all dogs studied. This body position and combination of leads is therefore suitable in evaluating electrocardiographic contour alterations in chronic experiments without concern over changes due to simple shifts in position of the dog's mobile heart.

As there are variations in contours encountered in different dogs (Fig. 3), each dog must serve as its own control. Such variation

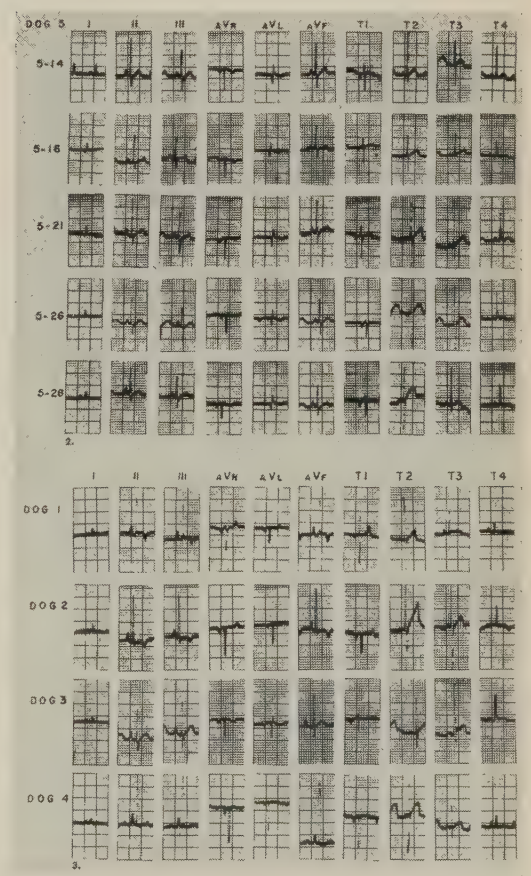


FIG. 2. Limb and chest leads of dog 5 taken on different days to show the fair degree of constancy in contour in the several leads. Discussed in text.

FIG. 3. Limb and chest leads in dogs 1 to 4 to show amount of variation seen among several dogs with leads employed. Discussed in text.

between subjects is also encountered in clinical electrocardiography.

Conclusion. A technic is described, and representative tracings shown, for taking serial electrocardiograms in anesthetized dogs. This technic gives virtually no contour changes in serial electrocardiograms and is therefore suitable for chronic experiments.

1. Katz, L. N., Soskin, S., Frisch, R., PROC. SOC. EXP. BIOL. AND MED., 1934, v32, 208.
2. Harris, B. R., Hussey, R., *Am. Heart J.*, 1934, v12, 724.
3. Gross, L., Benison, C., *ibid.*, 1937, v14, 677.
4. Peterson, E. S., Ricketts, N. R., Brewer, N. R., Lints, H. A., Test, C. E., Tupikova, N. A., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 330.
5. Lalich, J., Cohen, A. M., Walker, G., *Am. Heart J.*, 1941, v22, 105.

Received May 4, 1960. P.S.E.B.M., 1960, v104.

Properties of Diphtheria Antitoxins Produced in Guinea Pigs with Use of Freund's Adjuvant.* (25882)

SARA E. BRANHAM, C. W. HIATT AND DONALD B. RIGGS
Division of Biologics Standards, Nat. Inst. Health, Bethesda, Md.

In an earlier investigation of the toxins of several strains of *Corynebacterium diphtheriae*(1) we prepared antisera by inoculating guinea pigs with diphtheria toxoid incorporated in Freund's complete adjuvant. Antitoxins produced in this way were used in the conventional flocculation test(2) as well as in gel diffusion and immuno-electrophoresis experiments. The present report is concerned with the nature of the antibody and with protein composition of the sera as determined by zone electrophoresis on paper strips.

Materials and methods. Toxoids were prepared, as described previously(1), by incubating 1% solutions of dialyzed, lyophilized culture filtrates with 0.5% formalin for 6 weeks at 36°C. Three strains of *C. diphtheriae* were used to produce the toxins: the conventional PW 8 (type *intermedius*) and 2 strains of type *gravis*. For preparation of antisera, 6 groups of 500 g guinea pigs were selected. Each animal received 10 mg of toxoid (1 ml) mixed with 1 ml of Freund's complete adjuvant(3) containing *Mycobacterium butyricum* injected intramuscularly in the back of the neck. A 1-ml dose of the toxoid alone was inoculated into each animal at the same site one week later. Two weeks after second injection, the guinea pigs were bled, and the serum of each was titrated for antitoxin content by the guinea-pig skin test(4). Sera from animals that had received the same antigen and had approximately the same skin-test titer were pooled. Pooled serum from 20 uninoculated guinea pigs served as a control. The pooled antisera were re-titrated by skin test using Nat. Inst. of Health reference toxin #2562. L_f values of sera were obtained using flocculating toxin #8030, containing 60 L_f units per mg. Standard flocculating serum #F-6 was used as positive control. To obtain precipitation curves, various mixtures of PW-8 toxin and guinea-pig antitoxin were incubated for 1 hour at 37°C followed by 18 hours at 4°C, then measured for turbidity in a Fisher Nefluorophotometer. The protein composition of pooled antisera and of pooled normal guinea-pig serum was determined by paper-strip zone electrophoresis, using a Spinco Model R apparatus. Five or more replicate samples (0.006 ml) of each serum were allowed to migrate for 16 hours in Veronal buffer, pH 8.6, ionic strength 0.10, with a constant current of 2.5 ma per strip. The patterns were developed by staining with bromphenol blue and were scanned by an integrating densitometer. Protein nitrogen content of each serum was determined by a micro-Kjeldahl method and this value, multiplied by the factor 6.25, was taken as total

* The authors are indebted to Jacob E. Lieberman for statistical analysis of electrophoretic data and to Robert Blereau for nephelometric measurements.

TABLE I. Diphtheria Antitoxin Content of Guinea-Pig Sera as Determined by Skin Tests and Flocculation Reactions in 10 Serum Pools.

No. of guinea pigs in pool	Antigen used to immunize	Units specific antitoxin/ml (skin test)	L_f units antitoxin/ml
17	PW8	150	150
18	*(intermedius)*	200	200
33	*gravis* G 1	10	25
6		30	31
9	*gravis* G 2	20	
9		5	
10		5	31
10		25	25
15		5	
5		10	10
F6 (control)			240

protein content. The absolute concentration of each electrophoretic component was then computed and expressed as g per 100 ml of serum.

Results. The antitoxin content, determined by skin tests and flocculation reactions, is shown in Table I for each of the 10 serum pools. The PW8 preparation elicited a greater antibody response than either of the 2 *gravis* type toxoids. This was undoubtedly due to a greater amount of toxoid in the formalinized culture filtrate, for the PW8 strain is a copious toxin producer.

There was close agreement between skin-test end points and L_f values in 5 of the 8 serum pools upon which both tests were performed. In 2 of the pools (3 and 7) L_f values were considerably higher than skin test titers.

Precipitation curves for the reaction of PW-8 toxin with homologous guinea-pig antiserum (Fig. 1), indicate that there is inhibition in the region of antigen excess only. Thus the precipitins produced in the guinea pig in these experiments are of the "rabbit type"(5).

From the summary of electrophoresis data in Table II it may be seen that γ-globulin, and to some extent β-globulin is more plentiful in immune sera than in normal. Furthermore, the increase in γ-globulin has been at the expense of albumin and α-globulin fractions since total protein is essentially unchanged.

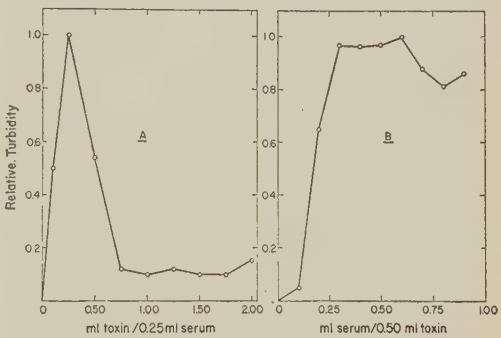


FIG. 1. Precipitation curves for reaction between diphtheria toxin (PW8) and homologous antiserum produced in guinea pigs. (A) Antibody held constant, antigen concentration varied. (B) Antigen held constant, antibody concentration varied.

If it is assumed that the alteration in protein composition is a non-specific response to the adjuvant,† and effects of the toxoids are ignored, then the 6 observations may be treated as replicate experiments which afford an estimate of mean values and standard deviations of protein components in the antisera. The estimates of standard deviation were made with 5 degrees of freedom, and the corresponding "t value" is 2.571.

The fiducial range for β-globulin in the an-

TABLE II. Protein Composition of Pooled Antisera Produced in Guinea Pigs by Inoculation with Diphtheria Toxoids and Freund's Adjuvant.

Inoculum		No. of animals	Albumin	α-glob.	β-glob.	γ-glob.	Total
			g/100 ml				
None		20	2.49	2.35	.77	.35	5.96
PW8 toxoid and Freund's adjuvant		10	2.20	1.78	1.06	.96	6.00
		10	1.93	2.19	1.15	1.29	6.56
G 1	*Idem*	10	2.06	2.23	1.25	.90	6.44
G 2	"	11	2.05	1.86	1.08	.98	5.97
		9	2.02	1.83	1.22	.89	5.96

† In a single animal injected with Freund's adjuvant alone, a similar elevation in γ-globulin was observed.

tisera is calculated to extend from 0.80 to 1.41. Since β-globulin in normal serum is found to be only 0.77, which is outside the fiducial limits, it may be inferred that the apparent increase in β-globulins, although slight, is statistically significant.

The 5% fiducial limits for γ-globulin component are 0.59 and 1.38. Since γ-globulin in the normal serum is well below the lower limit it is clear that the increase in γ-globulin is significant.

Antitoxin titers are not correlated with γ-globulin content. This tends to confirm the impression that the alterations in serum composition result from a non-specific response to the stimulus of Freund's adjuvant.

Summary. 1) Guinea pigs injected with diphtheria toxoid and Freund's complete adjuvant developed antibodies which neutralized the toxin in guinea-pig skin tests and formed visible precipitates in flocculation tests. Precipitation of the antigen-antibody complex was inhibited in antigen excess only, indicating that the guinea-pig antitoxin resembled that produced in the rabbit rather than the horse. 2) The γ-globulin content of pooled antisera, as determined by zone electrophoresis on paper strips, was significantly higher than that of normal guinea-pig serum.

1. Branham, S. E., Hiatt, C. W., Cooper, A. D., Riggs, D. B., *J. Immunol.,* 1959, v82, 397.

2. Nat. Inst. of Health, U.S.P.H.S., *Minimum Requirements: Tetanus Toxoid,* 4th Revision, 1952.

3. Freund, J., *Adv. Tuberc. Res.,* 1956, v7, 130.

4. Nat. Inst. Health, U.S.P.H.S., *Minimum Requirements: Diphtheria Antitoxin,* 2nd Revision, 1948.

5. Cohn, M., Pappenheimer, A. M., Jr., *J. Immunol.,* 1949, v63, 291.

Received May 4, 1960. P.S.E.B.M., 1960, v104.

Comparative Hemolytic Complement Activities of Germfree and Conventional Guinea Pig Serum. (25883)

WALTER L. NEWTON, ROBERT M. PENNINGTON AND JACOB E. LIEBERMAN
(Introduced by K. Habel)
National Institutes of Health, Bethesda, Md.

Fresh serum from normal guinea pigs is an excellent source of complement. It was of interest to determine whether serum of germfree guinea pigs also contained complement, and to compare its hemolytic activity with that of serum from conventional animals. Such studies could reveal whether contact with bacteria and parasites that normally contaminate the conventional animal is an important factor in determining serum complement level. As far as known, data on complement activity in germfree animals are limited to the following recent observations: Sera from 3 germfree rats each contained "50 units/ml" whereas sera from 3 conventional animals contained "65-80 units/ml"(1). A pool of sera from 6 germfree guinea pigs showed the same hemolytic complement activity as a pool from conventional animals of same age and strain(2).

Materials and methods. Germfree guinea pigs were obtained by Caesarean section of conventional pregnant animals from the NIH colony, and were maintained in Reyniers Germfree System units according to procedures and sterility checks described previously (3,4). Periodically, samples of fresh feces, food, bedding, etc., were examined microscopically and inoculated into various media. Failure to demonstrate presence of viable organisms constituted the basis for calling the animals "germfree." The diet consisted essentially of autoclaved mixture of oatmeal, commercial guinea pig pellets, kale, and carrots, to which vitamins were added(5). Each unit contained litters from 3 females, usually 9-10 guinea pigs. At 1, 2, and 3 weeks of age, 3 germfree (GF) guinea pigs, one from each litter, were removed, anesthetized immediately, and bled from the heart. Serum was separated from the clot by centrifugation and

stored at –70°C to minimize any possible loss of complement activity(6). Two groups of conventional animals obtained from same colony at age 2-4 days, were used as controls and maintained in animal room. One group (CV), was fed usual nonsterile stock diet of kale, carrots, and commercial guinea pig pellets. As a control for diet, the other group (CVD) was maintained on the same autoclaved ration given the germfree animals. Aside from containing living micro-organisms, controls also differed from germfree animals in that they were delivered normally and had suckled for a short period before being weaned. However, these variables have been difficult to control because it has not been possible to keep Caesarean-born, non-suckled guinea pigs alive for appreciable periods with any consistency outside the germfree system. Sera from 81 animals (9 each of GF, CV and CVD, at 1, 2, and 3 weeks of age) were collected over 3 months and tested simultaneously for complement activity, one month after last specimen was collected. Later, a second series, consisting of sera from 27 animals approximately 3 months old, was also examined to compare complement activity in more mature germfree and conventional animals. A modification of a technic(7) for determining 50% hemolytic complement activity, or K value, was used. This modification embodied use of spectrophotometer and increased the accuracy of determining the 50% hemolytic unit of complement.

TABLE I. Comparative Hemolytic Complement Activity of Germfree and Conventional Guinea Pig Serum.

Age in wk	Avg* amounts (ml) of 1:100 serum required to lyse 50% of sensitized sheep RBC's in 2% suspension = K values		
	Germfree (GF)	Conventional (CV)	Conventional on germfree diet (CVD)
1	1.40 (1.07-1.70)	1.26 (1.10-1.52)	1.77 (1.23-2.20)
2	1.06 (.90-1.18)	1.06 (.92-1.12)	1.15 (.90-2.10)
3	1.08 (.85-1.28)	.93 (.80-1.05)	1.24 (.68-1.65)
12	.64 (.49-.96)	.63 (.60-1.05)	.66 (.48-.83)

* Each avg based upon results with sera from at least 7, usually 9, animals. Figures in parentheses indicate range of values.

Results. The data shown in Table I were examined by an analysis of variance. It is obvious that there was no difference in hemolytic complement activity among 12-week-old animals. It is quite apparent that presence or absence of living micro-organisms is not a factor in determining complement levels.

Among younger animals, differences occurred between some groups. However, data were inconsistent in this regard. For example, average values of all 3 groups of 1-week-old animals differed significantly from each other ($P<.01$). Yet sera from the 3 groups of 2-week-old animals showed essentially the same activity. With the 3-week-old animals there were, again, significant differences among the groups ($P<.05$). It is difficult to interpret this variation, particularly since all sera of younger animals were tested at the same time with a single lot of reagents. However, the presence of living micro-organisms did not appear to affect complement levels. In fact, differences were usually larger between conventional animals on the 2 types of diet than between germfree and conventional animals.

All 1-week-old animals showed weaker complement activity than 3 adult animals tested concurrently. The latter, used as controls for test procedure and not shown in the Table, had K values ranging from 0.76 to 0.79, whereas values obtained with the youngest animals ranged from 1.07 to more than 2.20. Some complement levels were so low that K values had to be determined by extrapolation. This tended to reduce the accuracy of higher values and may have been a factor contributing to the variation encountered with younger animals. As a group, sera from 1-week-old animals had complement activity averaging only ⅓ to ½ of 12-week-old animals. We have not seen any published reference to this interesting age effect.

Summary. Hemolytic complement levels of more than 100 1-, 2-, 3-, and 12-week-old germfree and conventional guinea pigs were compared. There was no consistent significant difference between complement levels of

germfree and conventional animals, especially among older animals. All groups of 12-week-old animals had serum complement levels 2 to 3 times those of 1-week-old animals.

1. Wagner, M., *Ann. N. Y. Acad. Sci.,* 1959, v78, 261.

2. Toussant, A. J., Muschel, L. H., *Nature,* 1959, v183, 1825.

3. Reyniers, J. A., Trexler, P. C., Ervin, R. F., *Lobund Reports,* Univ. of Notre Dame, 1946.

4. Phillips, B. P., Wolfe, P. A., Gordon, H. A., *Ann. N. Y. Acad. Sci.,* 1959, v78, 183.

5. Warren, K. S., Newton, W. L., *Am. J. Physiol.,* 1959, v197, 717.

6. Osler, A. G., Strauss, J. H., Mayer, M. M., *Am. J. Syph., Gonor. & Ven. Dis.,* 1952, v36, 140.

7. Bozicevich, J., Hoyem, H. M., Walston, V. M., *Pub. Health Rep.,* 1946, v61, 529.

Received May 4, 1960. P.S.E.B.M., 1960, v104.

Role of Inhibitors in Hemagglutination Behavior of Mumps Virus.* (25884)

KARI CANTELL AND KARI PENTTINEN (Introduced by Thomas Francis, Jr.)
State Serum Inst., Helsinki, Finland

Mumps virus agglutinates red cells (rbc) of several animal species. However, it has been reported that the rbc of some species (1,2,3,4) are not agglutinable by mumps virus. Special attention has been paid to the inability of some mumps strains to agglutinate human rbc(5,6,7). According to Sohier *et al.* (7), 9 mumps virus strains fell into 2 groups as regards their hemagglutination (HA) behavior. The first group (Enders type), including 3 of the strains, agglutinated equally well fowl and human erythrocytes, the second group of 6 strains (Habel type) agglutinated human rbc only feebly or not at all. Sensitivity of mumps virus to HA inhibitors present in tissues and fluids of chick embryos is known(8,9). The role of these inhibitors in determining ability of some strains of mumps virus to agglutinate human rbc is the object of this study. It will be shown that all the studied virus strains (also Habel types) agglutinated human erythrocytes almost as well as fowl cells after elimination of inhibitors from the virus fluids.

Materials and methods. The following strains of mumps virus were employed in this study; number of passages undergone in this laboratory in the allantoic (all.) or amniotic (amn.) cavities of the embryonate egg are given in parentheses: Enders strain (68-149 all.), Habel strain (1-12 amn., 1-82 all.) obtained from Dr. V. J. Cabasso(6), KS strain (1-8 amn., 1-32 all.) isolated in this laboratory(10), Chopin strain (1-4 amn.) supplied by Dr. R. Sohier(7). For HA tests blood was taken only from fowls whose rbc were found to give uniformly high titers with mumps virus. Human erythrocytes were always taken from the same person of O blood group. Red cells washed 3 times were stored as a 10% suspension and used at a concentration of 0.5% in the assay procedure. Titrations were carried out at room temperature (18-20°C) on plastic plates by the pattern method(11) and read after 2 hours. Amniotic and allantoic fluids containing virus were treated by various procedures to free them of inhibitor activity. Some were dialyzed for 24 hours against phosphate buffered saline (pH 7.4) at 4°C; others were diluted 5-fold and sonicated for 30 minutes at ice temperature (20 KC. MSE Mullard) while some were merely incubated 2 days at 35°C. Enzymatic treatment was effected by incubating at 37°C for 18 hours a mixture of 2 parts of cholera filtrate (Philips-Roxane) and one part of fluid containing virus. In all HA titrations of the virus preparations treated with cholera filtrate, sodium citrate was added to the reagents to give a final concentration of one per cent. To remove inhibitors by differential centrifugation, one ml of virus fluid and 9 ml of buffered saline were combined and centri-

* Supported by grant from Sigrid Juselius Fn.

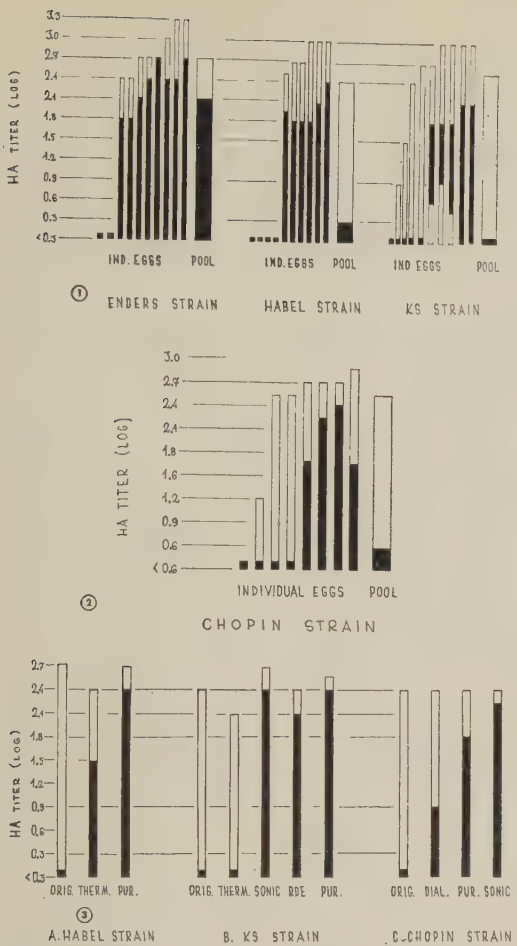


FIG. 1. HA titers with fowl and human red cells of individual and pooled allantoic fluids infected with various mumps strains.

FIG. 2. HA titers with fowl and human red cells of individual and pooled amniotic fluids infected with Chopin strain of mumps virus.

FIG. 3 (A, B and C). Effect of various treatments of virus preparations on their capacity to agglutinate human red cells. ORIG. = original virus preparation; THERM. = incubated for 2 days at +35°C *in vitro*; DIAL. = dialysed overnight against buffered saline; PUR. = partially purified by ultracentrifugation; SONIC = treated in ultrasonic disintegrator; RDE = treated with cholera filtrate. Details in text.

fuged (Spinco model L) for 60 minutes at 26, 530-56, 550 x G. The supernatant was discarded and the sediment suspended in one ml of buffered saline.

Results. In our first passages of the Habel strain, it behaved as described by Cabasso and Cox(6), *i.e.*, it did not agglutinate human rbc at any temperature. During the first amniotic passages, however, some virus fluids had a slight ability to agglutinate human erythrocytes. When the strain was passed allantoically, virus fluids giving high HA titers with human rbc began to appear rather frequently. The same was found with the KS strain. Conversely, with the Enders strain it was seen that some low titered virus preparations lacked ability to agglutinate human rbc. These observations suggested that this ability was influenced by some extrinsic factors. Further information was obtained by determining HA titers in individual infected eggs. A typical experiment of this type is given in Fig. 1. About 10^4 infective doses of Enders, Habel and KS strains were each inoculated into 10 eggs allantoically. After 5 days' incubation at +35°C the allantoic fluids were collected separately and the HA titer of each one was determined with fowl and human rbc. Also equal amounts of the allantoic fluids from individual eggs were pooled and HA titers determined. The figure shows that with the Enders strain the pool titers corresponded quite well with those of individual eggs. The Habel strain when pooled lost the ability to agglutinate human rbc almost entirely, while the titer with fowl cells remained high. With the KS strain, the reciprocal ratio of fowl titer and human titer in individual eggs varied greatly. Some infected allantoic fluids agglutinated human cells rather well, others only patchily (a phenomenon often seen in HA titrations with human erythrocytes), while others not at all, in spite of their high titers with fowl cells. The pool gave a high titer with fowl cells but no agglutination at all with human cells.

In the following experiments different methods were used to remove the activity of HA inhibitors which might be present in the virus preparations and then HA titers obtained with human and fowl cells were compared. These experiments included the Chopin strain which has been reported to agglutinate very weakly human rbc(7). In our laboratory pooled amniotic fluids infected with this strain gave the same results but some individual amniotic fluids agglutinated human erythrocytes to high titer (Fig. 2).

In Fig. 3 (A, B, and C) are shown HA titers obtained with fowl and human cells using preparations of mumps virus treated in various ways. All virus fluids included in this experiment originally failed to agglutinate human cells. However titers with these cells were not far below those with fowl cells once the virus fluids were treated with ultrasonic waves (B, C) or cholera filtrate (B) or partially purified by ultracentrifugation (A, B, C). Slight ability to agglutinate human rbc was revealed in the Chopin strain by dialysis against buffered saline (C). Thermal treatment (2 days at +35°C *in vitro*) of the Habel strain (A) but not of the KS strain (B) also brought out some human cell agglutination.

Discussion. According to Buzzel and Hanig(9) the HA behavior of mumps virus is affected by its heavy contamination with inhibitors, which by giving the virus a strong negative charge hamper attachment to the red cell. On the other hand, it is known that the inhibitor in normal allantoic fluid affects to greater degree the attachment of mumps virus to human than fowl rbc(12,5). From this, one could draw the conclusion that the ratio between fowl titer (F) and human titer (H) of a given mumps virus preparation is directly proportional to inhibitor contamination. Our results are consistent with this conclusion. The methods which raised the F/H ratio of the virus preparations destroyed or removed inhibitors(9,13). Inversely, the pooling of individual egg fluids tended to depress the F/H ratio by incorporating the inhibitors from low titer and negative eggs, which are typically present in mumps infected embryonated eggs(10). Variations of F/H ratio in individual eggs may be explained as also being caused by inhibitors, bearing in mind the above-mentioned variation in production of hemagglutinins by individual eggs and the differences in amount of inhibitors in individual allantoic fluids(8,14).

Obviously contamination of mumps virus by inhibitors is not an irreversible process but a balanced condition where the essential factors are virus, inhibitor, and rbc(14,15). When this system of 3 factors prevails variations in HA results are to be expected depending on quality and concentrations of these factors.

The results obtained disagree with the supposition that certain mumps virus strains are characterized by certain F/H ratios. In fact, F/H ratio could vary in individual eggs infected simultaneously and also in different pooled virus fluids. However, the Habel and Chopin strains generally gave higher F/H values than the Enders strain. The inhibitor sensitivity of different strains and their ability to destroy them thus still demand further elucidation.

Summary and conclusion. 1) Low ability of some strains of mumps virus to agglutinate human rbc was studied. Some individual-infected amniotic and allantoic fluids and pooled virus preparations agglutinated human erythrocytes nearly as well as fowl erythrocytes. When virus fluids, failing to agglutinate human cells, were treated by various procedures capable of removing or destroying inhibitors, they became positive. 2) Failure to agglutinate human erythrocytes is not a stable intrinsic characteristic of certain strains of mumps virus but is due to inhibitors present in virus preparations.

1. Tamm, I., *J. Immunol.,* 1954, v73, 180.

2. Chu, L. W., Morgan, H. R., *J. Exp. Med.,* 1950, v91, 403.

3. Enders, J. F., *Harvey Lect.,* 1947-48, v43, 92.

4. Borecky, L., *Folia Biol.* (Praha), 1953, v2, 344.

5. Florman, A. L., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 279.

6. Cabasso, V. J., Cox, H. R., *ibid.,* 1955, v88, 370.

7. Sohier, R., Favier, G., Chardonnet, Y., Challut, F., *Ann. Inst. Pasteur,* 1956, v91, 137.

8. Beveridge, W. I. B., Lind, P. E., *Austr. J. Exp. Biol. Med. Sci.,* 1946, v24, 127.

9. Buzzel, A., Hanig, M., *Advances in Virus Research,* 1958, v5, 289.

10. Cantell, K., *Ann. Med. Exp. Fenn.,* 1959, v37, Suppl. 1.

11. Salk, J. E., *J. Immunol.,* 1944, v49, 87.

12. Lind, P. E., *Austr. J. Exp. Biol. Med. Sci.,* 1948, v26, 93.

13. Penttinen, K., *Ann. Med. Exp. Fenn.,* 1954, v32, 76.

14. Hardy, P. H., Horsfall, F. L., Jr., *J. Exp. Med.,* 1948, v88, 463.

15. Penttinen, K., Tommila, V., Cantell, K., *Ann. Med. Exp. Fenn.,* 1955, v33, 159.

Received May 6, 1960. P.S.E.B.M., 1960, v104.

Effect of Underfeeding on Thyroxine Secretion Rate of Female Mice.* (25885)

G. W. Pipes, J. A. Grossie and C. W. Turner†
Dept. of Dairy Husbandry, University of Missouri, Columbia

It is generally recognized that underfeeding reduces thyrotropin (TSH) secretion and thyroid function. However, quantitative data on effect of underfeeding on thyroxine (T4) secretion rate is not available. Development of a method of determining thyroxine secretion rate (TSR) in mice(1,2) makes such studies possible.

Materials and methods. Adult female Swiss Webster (Beyer strain) mice, kept at temperature of 78 ± 1°F were used. Normal mice were found to consume an average of 1.3 g/10 g body weight/day of Purina Lab. Chow. Other groups received 75 and 50% of this amount of feed daily for 16 days. Each mouse then received i.p. 6 μc I^{131}. Three days later, whole body count was made at a distance of 2 in. Then L-thyroxine was injected at rate of 0.25 μg/100 g/day. At 2 day intervals counts were repeated. If thyroidal-I^{131} was not blocked (95-100% of previous count) 0.5 μg/100 g T4 was injected with further increments of 0.5 μg/day until TSR was reached. Conventional corrections were made for background and I^{131} decay.

Results. The control group showed a slight but not significantly higher mean TSR in comparison with previous study(1). Body weight was not altered on ¾ full feed, but was reduced 17.3% on ½ feed (Table I). Mean TSR on ¾ feed was reduced significantly to 0.93 μg/100 g/day or 33% in comparison with control group, whereas the ½ fed group showed a further reduction to 0.80 μg/100 g/day or 41%.

Discussion. Indirect evidence of reduced thyroidal function resulting from varying degrees of underfeeding such as thyroidal uptake of I^{131}(3,4) lowered levels of PBI(5) and depressed rate of release of thyroidal-I^{131} (6) have been presented. These have been shown to be poor indices of thyroidal function in comparison to determination of TSR(7). The present study indicated that ¾ full feed significantly decreased TSR (33%) without an effect upon body weight. These observations indicate that pituitary-thyroid axis is sensitive to mild underfeeding resulting in reduced TSR sufficient to maintain body weight by a rapid reduction in BMR.

This degree of underfeeding should not be considered a stressful situation but rather a rapid adjustment of thyroxine secretion to meet the environmental problem of reduced energy intake.

Upon reduction of feed intake to ½ normal, further reduction of TSR 41% associated with a decrease in body weight might produce stress. The possible influence of ACTH and increased release of glucocorticoids under such conditions upon thyroid function is unresolved. Several workers have reported reduced thyroidal-I^{131} uptake when ACTH or cortisone was administered(8,9,10, 11) but the effect upon release rate has varied from no effect(9,10), increased effect(12), to reduced effect(13).

Study of the influence of these hormones upon TSR will aid in resolving this problem. If it is shown that ACTH and the glucocorticoids depress TSR, it is believed present data indicate such an effect occurs only upon severe underfeeding (½) and is secondary to direct depression of TSH secretion caused by underfeeding.

Summary. Adult female Swiss Webster mice kept at temperature of 78°F were observed to eat 1.3 g/10 g body weight/day of Purina Lab. Chow. Their daily L-thyroxine secretion rate (TSR) was 1.38 ± .173 μg/100 g body weight. Mice fed ¾ ration for 16 days showed TSR of 0.93 μg/100 g/day, a significant reduction of 33%, yet no loss in body weight occurred. On ½ ration the TSR

* Contribution from Mo. Agr. Exp. Sta. Journal Series No. 2157. Approved by Director.

† This investigation was supported in part by a grant from U. S. Atomic Energy Commission.

TABLE I. Effect of Underfeeding on Thyroxine Secretion Rate in Female Mice.

Treatment, rate of feeding	No. of animals	Body wt, g Initial	Final	TSR, μg/100 g/day	Decrease, %	Frequency distribution of TSR .25 μg	.5 μg	1 μg	1.5 μg	2.0 μg	2.5 μg
Control *	58			1.25 ± .05			3	30	18	7	
Control 2 †	16	30.3	30.4	1.38 ± .173	100		4	3	4	3	2
¾ 3 ‡	14	28.5	30.1	.93 ± .180	33	2	7		3	1	1
½ 4 §	13	31.0	24.8	.81 ± .142	41	2	6	1	4		

* Wada *et al.*(2). † 2 *vs* 3 $p < .05$. ‡ 2 *vs* 4 $p < .01$. § 3 *vs* 4 not significant. 1 *vs* 2 not significant.

was reduced to 0.80 μg/100 g/day or 41% associated with a 17.3% reduction in body weight. These data suggest that pituitary-thyroid axis is sensitive to slight reduced energy intake by reduction in TSR sufficient to maintain body weight.

1. Amin, A., Chai, C. K., Reineke, E. P., *Am. J. Physiol.,* 1957, v191, 34.

2. Wada, H., Turner, C. W., PROC. SOC. EXP. BIOL. AND MED., 1959, v102, 608.

3. Meites, J., Wolterlink, L. F., *Science,* 1950, v111, 1755.

4. Meites, J., *Iowa State College J. Sci.,* 1953, v28, 19.

5. Blincoe, C., Brody, S., *Mo. Agr. Exp. Sta. Res. Bull.* 576, 1955.

6. Reichlin, S., *Endocrinology,* 1957, v60, 470.

7. Turner, C. W., Pipes, G. W., Premachandra, B. N., Conference on Isotopes in Agr., Okla. TID-7578, 97, 1959.

8. Money, W. L., Kraintz, L., Foger, J., Kirschner, L., Rawson, R. W., *Endocrinology,* 1951, v48, 682.

9. Perry, W. F., *ibid.,* 1951, v49, 284.

10. Albert, A., Tenney, A., Ford, E., *ibid.,* 1952, v50, 324.

11. Myant, N. B., *J. Physiol.,* 1953, v120, 288.

12. Beck, R. N., *Endocrinology,* 1958, v62, 9.

13. Brown-Grant, K., *ibid.,* 1955, v56, 607.

Received May 9, 1960. P.S.E.B.M., 1960, v104.

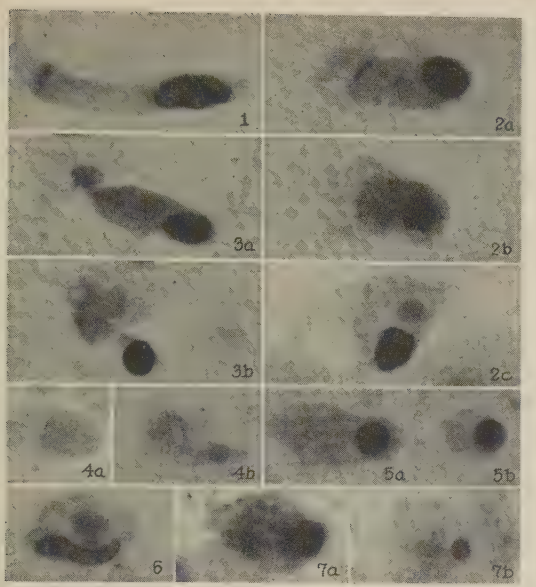
Ciliocytophthoria in Sputum from Patients with Adenovirus Infections.*† (25886)

CYNTHIA H. PIERCE AND ALICE W. KNOX (Introduced by James G. Hirsch)
Rockefeller Inst. and Dept. of Microbiology, College of Physicians and Surgeons, Columbia University, N. Y. C.

Papanicolaou(1) described morphologic abnormalities in ciliated epithelial cells exfoliated from the respiratory tract of certain patients with acute or chronic pulmonary disease. He designated this phenomenon ciliocytophthoria (CCP). Further observations in our laboratory established an association of CCP with virus infections of the human respiratory tract(2). CCP was found to be present in persons suffering from viral infections but absent in those with bacterial pulmonary disease. Of the viral illnesses studied in which CCP was observed, only those due to influenza were documented by laboratory data (*i.e.* virus isolation or rise in serum antibody). The remainder were classified on the basis of clinical and laboratory findings as diseases of probable viral etiology. The present report presents data derived from screen-

* Aided by grant from Am. Cancer Soc.; these studies also were conducted under auspices of Commission on Influenza, Armed Forces Epidemiological Board and supported by Office of Surgeon General, Dept. of Army, Washington, D.C.

† This investigation has been carried out with cooperation of many individuals. We should like to express appreciation to Dr. George Papanicolaou for continued interest and counsel in cytologic studies, to Dr. Harry M. Rose for the correlation with immunologic studies, and to Col. Joseph W. Cooch and his staff at Fort Dix Health Center, N. J., for supplying specimens. Also to Drs. Purnell Choppin and Suydam Osterhout, Hosp. of Rockefeller Inst., and acknowledge technical assistance of Sylvia J. Fullerton and Marilyn J. Haikara, Rockefeller Inst.



Morphologic changes occurring in Ciliocytophthoria (CCP), magnification × 850, Papanicolaou stain 1-6, Mallory stain 7.

FIG. 1. Normal ciliated epithelial cell.

FIG. 2. Intact ciliated epithelial cells with abnormalities: a. Pyknotic nucleus and cytoplasmic inclusions. b. Nuclear and cytoplasmic inclusions. c. Chromatin marginated at periphery of nucleus. One large and many small cytoplasmic inclusions.

FIG. 3. Pinching off of ciliated portions resulting in: a. Small ciliated tuft and large basal segment containing pyknotic nucleus and cytoplasmic inclusions. b. Large ciliated tuft and small basal segment containing pyknotic nucleus and one small cytoplasmic inclusion.

FIG. 4. Free anuclear ciliated tufts: a. Homogeneous cytoplasm with one weakly stained inclusion. b. Vacuolated cytoplasm with one large deeply stained inclusion.

FIG. 5. Basal nucleated segments: a. Pyknotic nucleus and several cytoplasmic inclusions. b. Nuclear inclusion and large cytoplasmic inclusion.

FIG. 6. Phagocytosis by macrophage of an anuclear segment containing large inclusion.

FIG. 7. Mallory stained preparations: a. Intact ciliated epithelial cell with pyknotic nucleus and many cytoplasmic inclusions. (Nuclear inclusion out of focus, visible only upon refocus.) b. Anuclear ciliated tuft containing large inclusion.

ing sputum specimens obtained from armed forces personnel at Fort Dix. The results demonstrate the presence of CCP in persons suffering from documented adenovirus infections and also confirm occurrence of CCP during viral influenza.

Methods. Through the courtesy of Col. Joseph W. Cooch, Health Center, Fort Dix, N. J., single sputum specimens were obtained from 375 recruits exhibiting symptoms of respiratory disease during March 4 to Sept. 9, 1958. Each specimen was stained according to the Papanicolaou technic(3) and examined microscopically for presence of CCP using criteria previously reported(2). In a small series of CCP positive specimens duplicate smears were made and stained with Mallory's aniline blue collagen stain(4) (acid fuchsin 30 seconds, aniline blue solution 50 seconds, followed by gradual dehydration). Paired acute and convalescent sera obtained from 86 of these patients were titrated for adenovirus antibodies in complement fixation (CF) tests. In addition, sera from 32 of the 86 patients were similarly examined for antibodies against influenza A. The prevalence of adenoviral infection among recruits during the period under study was determined by assaying complement fixing antibodies in individually paired serum specimens from a total of 578 patients. Immunologic tests were done with minor modifications of a previously described technic(5). A 4-fold or greater increase in titer of the convalescent serum as compared with that of the acute phase specimen was used as criterion of infection.

Results. The accompanying photomicrographs demonstrate the abnormal morphologic changes characteristic of CCP. The typical orange-pink color of the acidophilic inclusions obtained with the Papanicolaou stain can be seen in photomicrographs previously published(2). In Mallory-stained smears, cytoplasmic inclusions are a brilliant cherry red and nuclear inclusions are orange. Although the Mallory technic of staining as employed in our laboratory does not allow as good preservation of cells as does the Papanicolaou method, the rapidity and simplicity of the former procedure make it valuable in certain instances.

Among a total of 357 expectorated samples, 282 were deep cough specimens suitable for examination. CCP was observed in 59% of the specimens (Table I). No evidence of CCP was found in 30%, and in 11% there were abnormal elements suggestive of CCP, but not conclusive. In such instances small red-staining spherical bodies and mononuclear cells predominated in the sputum smear. It

TABLE I. Occurrence of CCP in Single Deep Cough Specimens from 282 Military Recruits Suffering from Acute Respiratory Disease.

CCP	No. of specimens	% of total
Positive	167	59
Negative	85	30
Abnormal	30	11

is uncertain whether or not this pattern represents a transition phase between the early stage of viral infection, when CCP is readily recognized, and the later stages of infection, or convalescent period, when large multinucleated histiocytes with deeply staining orange cytoplasm are the principal cellular elements.

During CCP observation period, 578 paired serum specimens were examined for presence of adenovirus antibodies by complement fixation (CF). Incidence of patients with CCP was compared with the prevalence of adenovirus infection at Fort Dix during monthly intervals throughout the survey. The results shown in Table II demonstrate that a relatively high percentage of sputum samples were positive for CCP during each month, and that concomitantly there was a high incidence of adenovirus infection, as demonstrated serologically.

The direct comparison of cytologic findings with adenovirus antibody studies in 86 patients from whom sputum specimens as well as acute and convalescent sera were obtained is shown in Table III. Sera from 32 of these individuals (Group A) also were examined for increased titers against influenza A virus. Among this group were 27 positive CCP specimens. Twenty-two of the CCP positive cases occurred in persons who had a rise in adenovirus antibody titer, 3 showed increases in influenza antibody, and one revealed serologic evidence of both adenovirus and influenza infection. Only one case was CCP positive without a demonstrable rise in antibody titer. Single sputum specimens from 5 individuals with serum antibody rises against adenovirus failed to show CCP. Among the 54 patients comprising group B, CCP was observed in 33, 25 of whom showed significant rises in adenovirus antibodies. All 6 persons whose sputum smears were abnormal, but not classified as CCP, had adenovirus infection as documented serologically.

A summary of the foregoing data with respect to adenovirus infection shows that from 60 patients having positive CCP specimens, 48 had significant adenovirus antibody rises as did all 6 individuals presenting abnormal specimens.

Discussion. The finding of CCP in 59% of 282 single sputum specimens indicates that this phenomenon is by no means uncommon among military recruits exhibiting symptoms of respiratory disease. Whether other population groups would yield such a large percentage of CCP positive specimens cannot be determined from the present study. Data from the immunologic studies serve to establish that CCP is associated with adenovirus infection as well as with influenza. In addition, the results confirm the high incidence of adenovirus disease among military personnel at Fort Dix as previously reported(6).

Since the cytologic observations here reported were obtained by examining only a single deep cough specimen from each patient, the negative findings associated with positive serologic evidence of adenovirus infection might have been reversed had several sputum

TABLE II. Incidence of CCP and Adenovirus Infection during Survey Period.

Test periods during 1958	CCP					Significant adenovirus antibody rise		
	No. patients	Pos.	Abn.	Neg.	% pos.	No. patients	No. pos.	% pos.
March	76	48	4	24	63	279	220	79
April	75	45	6	24	60	84	72	86
May	41	23	8	10	56	53	48	91
June	47	25	4	18	53	42	38	91
July	16	7	3	6	44	31	25	81
August	17	13	1	3	76	43	22	51
September	10	6	4	0	60	46	32	70

TABLE III. Comparison of CCP and CF Antibody Tests.

	Total No. individuals	Presence of CCP		Significant antibody rise: Adenovirus +	Adenovirus 0	Influenza A +	Influenza A 0
A.	32	Positive	22	22	0	0	22
			3	0	3	3	0
			1	1	0	1	0
			1	0	1	0	1
		Negative	5	5	0	0	5
B.	54	Positive	25	25	0		
			8	0	8		
		Abnormal	6	6	0	Not done	
		Negative	9	9	0		
			6	0	6		

samples from each individual been analyzed. Moreover, the possibility of a superimposed bacterial infection cannot be excluded in such instances, since it has already been observed that influenza patients with secondary bacterial infection no longer produce positive CCP specimens(2).

Examination of sputum specimens for CCP is a rapid laboratory test to distinguish viral infections of the respiratory tract from bacterial disease when used in conjunction with other laboratory procedures(2). The Papanicolaou stain, however, has not yet revealed significant differences between viral species in respect to their effect upon ciliated epithelium exfoliated from the respiratory tract. Other workers have shown that the finding of abnormal changes in exfoliated respiratory epithelium is an aid in establishing a tentative diagnosis of influenza(7,8). In addition morphologic changes in respiratory epithelium *in situ* have been observed upon examination of autopsy material obtained from fatal cases of influenza(7) and adenovirus infection(9,10).

The present results demonstrate the association of CCP with both adenovirus and influenza disease and support the concept that CCP may occur in a variety of viral respiratory infections.

Summary. A total number of 282 single sputum specimens from patients with acute respiratory disease were stained by Papanicolaou technic and examined for presence of certain abnormalities, known as ciliocytophthoria (CCP), in exfoliated ciliated epithelial cells. Fifty-nine % yielded positive results, 30% were negative, and 11% inconclusive. During this period complement fixation (CF) tests carried out on paired acute and convalescent sera from 578 patients demonstrated high incidence (77%) of adenovirus infection. The direct association of CCP with adenovirus infection was established by study of patients from whom sputum as well as serum specimens were obtained. Significant adenovirus antibody rises occurred in 48 (80%) of 60 individuals whose sputum specimens were positive for CCP.

1. Papanicolaou, G. N., *N. Y. State J. Med.,* 1956, v56, 2647.

2. Pierce, C. H., Hirsch, J. G., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 489.

3. Papanicolaou, G. N., *Atlas of Exfoliative Cytology,* Harvard Univ. Press, 1954.

4. Kolmer, J. A., Boerner, F., *Approved Laboratory Technic,* D. Appleton-Century Co., New York, 1945.

5. Lennette, E. H., Clark, W., Dean, B. H., *Am. J. Trop. Med.,* 1949, v29, 527.

6. Hilleman, M. R., Anderson, S. A., Levinson, D. J., Luecking, M. L., *Am. J. Hyg.,* 1958, v67, 179.

7. Hers, J. F. Ph., *Histopathology of the Respiratory Tract in Human Influenza,* H. E. Stenfert Kroese, N. V., Leiden, 1955.

8. Pigarevski, V. E., *Acta Virol.* (English Edition), 1957, v1, 30.

9. Chany, C., Lépine, P., LeLong, M., Le-Tan-Vinh, Satgé, P., Virat, J., *Am. J. Hyg.,* 1958, v67, 367.

10. Kusano, N., Kawai, K., Aoyama, Y., *Jap. J. Exp. Med.,* 1958, v28, 301.

Received May 9, 1960. P.S.E.B.M., 1960, v104.

A Simple Tissue Culture Maintenance Medium Utilizing Egg White. (25887)

L. F. Guerin and S. F. Kitchen (Introduced by F. P. Nagler)
Virus Labs., Lab. of Hygiene, Dept. Nat. Health and Welfare, Ottawa, Canada.

In a search for an efficient maintenance medium that would be simple to prepare and composed of readily available ingredients, it was decided to explore the practicability of a preparation containing albumin from avian egg. Antibacterial activity of egg white has been recognized for half a century, though the precise mechanism is not understood. It is also known(1,2) that antibodies may be transmitted from the female bird to the young through the egg, though their location in the latter is fortunately in the yolk(3). It remained to determine whether egg albumin could satisfactorily sustain developed tissue cultures in a healthy state for appreciable periods and whether it would exert any antiviral action against the enteroviral group of agents.

Material and methods. Egg white was separated from yolk of 12 fresh domestic hen's eggs under aseptic conditions and placed in sterile 250-ml centrifuge bottle containing a few glass beads. It was cooled and agitated in mechanical shaker for 10 minutes at 4°C, then frozen and thawed twice, inactivated at 56° for 30 minutes and finally centrifuged at 2000 rpm for 20 minutes. Three distinct layers were evident following centrifugation, *viz.*, a foamy upper, clear middle and dense, opaque lower layer. The clear middle layer, comprising about 80% of volume, was removed and used without further treatment; hereinafter it will be referred to as EA or albumin, although it is realized that egg white contains other chemically identifiable entities as well. The maintenance value of albumin was tested on cultures developed from freshly trypsinized monkey kidney and rabbit kidney, and on grown out cultures of 2 established cell lines, *viz.*, human amnion (FL) and our own human kidney line (HK/55). Egg albumin-saline (EAS) mixtures, containing 5%, 10% and 20% of albumin, were prepared and used as maintenance media, replacing the M150(4) and 10% calf serum (CS) after cell sheets had developed. Two variations were introduced as follows: (1) in half of primary tissue cultures and in half of established line subcultures, 10% EAS was substituted for usual growth medium (M150 plus 10% CS serum) when cultures were prepared, and (2) in half of all cultures the medium was renewed every 3 to 5 days; in the other half it remained unchanged. All cultures were observed for 2 to 3 weeks. Examination of our EA preparation for presence of antienteroviral factors was carried out in 2 ways. First, 38 cytopathogenic members of that group of agents, including the 3 poliovirus types, Coxsackie A-9 and B types 1-6 and Echo types 1-28 were exposed to 20% EAS for 90 minutes at room temperature (RT), with final virus concentration at approximately 100 $TCID_{50}$. Monkey kidney cultures were inoculated with virus mixtures, and 20% EAS was used as maintenance medium. This experiment was repeated with 4 separately prepared lots of albumin. Parallel virus control mixtures were also included, using M150 with and without 4% CS instead of 20% EAS. Secondly, monkey kidney cultures were inoculated with 3 enteroviruses, type I poliovirus, type B-1 Coxsackie and type I Echo virus, each diluted to 10^{-4}. When degeneration was complete, subcultures were made, diluting the fluid to 10^{-4}. In all, 3 successive subcultures were thus made, with EAS serving as maintenance medium in the original culture and 3 subcultures. Finally, the fluid from last serial culture was titrated for each virus in parallel using M150 (control) and 20% EAS as diluents. To test the practical value of EAS during viral isolation 25 known virus-bearing faecal or other clinical specimens were reexamined. Specimens represented the poliovirus (3 types), Coxsackie B (2 types) and Echo (one type) subgroups of viruses. Parallel monkey kidney cultures were inoculated, utilizing in one case

20% EAS and in the other (control) M150 as maintenance media. This experiment also provided additional evidence concerning possible presence of antiviral substances in albumin. Furthermore, we noted antibacterial activity of albumin. In 2 specimens bacterial contaminants that had apparently escaped preliminary treatment appeared in control cultures containing M150, obviously resistant to antibiotics (penicillin 100 i.u./ml and streptomycin 100 μg/ml) we normally employ in that medium. The 2 organisms were identified as *Alkaligenes fecalis* and *Aerobacter aerogenes*. Heavy broth cultures of each were prepared and each culture was titrated out to 10^{-6} in decimal dilutions in M150 (control) and M150 containing 5% EA. Adaptation of EA to use in plaque formation was attempted as follows: 89 ml of 1.7% NaCl solution (autoclaved and cooled), 10 ml EA and 1 ml of 0.2% neutral red solution (autoclaved and cooled) were mixed and pH reduced to 8 with *N* HCl. To aliquots of this mixture (at 40°C) were added equal quantities of 3% agar that had been autoclaved and cooled to 40°C. This was then poured over monkey kidney cultures already inoculated with the 3 types of poliovirus. The cultures were incubated at 35°C.

Results. The value of EAS as a maintenance medium proved quite satisfactory. With it the designated tissue cells survived in good condition for 7-10 days; renewal of fluid every 3-5 days insured survival for 2 weeks or longer. Use of EAS during isolation attempts also proved advantageous. Cytopathic changes appeared earlier in 13 instances with EAS than in M150 controls, on the same day in 10 cases, but never later with EAS.

During various experimental uses, 5% EAS was as effective as 20% EAS. In addition to serving as a maintenance medium, EAS supported growth of trypsinized second passage monkey and rabbit kidney cells, but not that of primary cultures of those tissues.

Although the 2 bacterial contaminants were resistant to penicillin and streptomycin, they did not appear in tissue cultures covered with M150 containing 5% albumin. The latter, while completely inhibiting *Alkaligenes fecalis*, was unable to suppress *Aerobacter aerogenes* in the lower 3 dilutions of the decimal titration series; it did so in the subsequent dilutions, however. Antibiotics in M150 controls were unable to inhibit either bacterial agent in any dilution.

Exposure of 38 enteroviruses to 20% albumin for 90 minutes at RT did not appear to have any more inhibiting effect than did the M150 without calf serum, as evidenced by time required to induce cytopathic effects. Antiviral activity of M150 with 4% CS, however, was observed in the case of each of 3 separate lots of CS represented. M150 with CS from Lot 1 inhibited for at least 7 days the production of cytopathic changes by Coxsackie B-5, Echo-1 and E-3; with CS from Lot 2 similar inhibition of E-12 and E-20 was noted, and with CS from Lot 3, cytopathogenic action by E-2 and E-23 was inhibited. Results in the titration experiment showed no significant difference in final titre between viruses that had been subcultured and titrated in 20% EAS and those subjected to M150 (controls).

Plaques formed by polioviruses using EAS-agar mixture were at least as large and as numerous as those observed when conventional and more elaborate salt-protein-agar mixtures were used.

EA appeared equally effective following lyophilization and reconstitution as it had been prior to this treatment.

Discussion. It is apparent from these results that albumin from domestic hen's egg, following simple preliminary treatment, can be utilized to serve several useful purposes in the application of current tissue culture technics to virology. When combined with physiological saline, not only is it effective as a maintenance medium during periods usually required either for viral isolation attempts or neutralization tests, but it also contains factors enabling second passage cells of monkey and rabbit kidney to establish themselves *in vitro* and grow. The albumin was unable to replace calf serum for outgrowth of primary cell cultures; unlike calf serum it was

not demonstrated to possess any antienteroviral substances.

Two beneficial characteristics of albumin were evident during its use in applied, or diagnostic, virology. First, the tendency toward earlier appearance of cytopathic changes observed when EAS was used, as compared with M150, may well be attributable to the relatively high pH (about 8.5) which characterizes EA and which the latter imparts to EAS. That acid reaction in tissue culture medium exerted an adverse effect on cytopathogenicity of certain viruses ("pH effect") and that this could be overcome by neutralization of the acid was pointed out by Barron and Karzon (5). Secondly, the antibacterial factor in EA is a particular advantage since bacterial contaminants occasionally escape preliminary centrifugation and antibiotics to which fecal specimens are subjected. It was obvious that the EAS medium was able at least to suppress multiplication of 2 bacterial agents in isolation cultures, as described, although 2 commonly used antibiotics were unable to do so. The active agent in albumin may be lysozyme (6).

Ability of EAS to serve as substitute for calf serum-balanced salt solution in agar overlay used for plaque formation was advantageous; so also was survival of its attributes following lyophilization and reconstitution.

Summary. Albumin from domestic hen's egg can be simply adapted for use as tissue culture medium for certain purposes. Combined with physiological saline it supports outgrowth of secondary cultures of monkey and rabbit kidney cells; it also sustains grown out cultures of fresh tissue and established cell lines for 2 weeks. Albumin possesses antibacterial properties, but no demonstrable antienteroviral activity. It can be lyophilized and reconstituted without loss of attributes.

1. Reeves, W. C., Sturgeon, J. M., French, E. M., Brookman, B., *J. Inf. Dis.,* 1954, v95, 168.

2. Kissling, R. E., Eidson, M. E., Stamm, D. D., *ibid.,* 1954, v95, 179.

3. Jukes, T. H., Fraser, D. T., Orr, M. D., *J. Immunol.,* 1934, v26, 353.

4. Morgan, J. F., Campbell, M. E., Morton, H. J., *J. Nat. Cancer Inst.,* 1955, v16, 557.

5. Barron, A. L., Karzon, D. T., PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 393.

6. Gottschalk, A. Lind, P. E., *Brit. J. Exp. Path.,* 1949, v30, 85.

Received May 9, 1960. P.S.E.B.M., 1960, v104.

Clearance of Blood Coagulation Product I in Rabbits.* (25888)

THEODORE H. SPAET AND JOSE CINTRON
Dept. of Hematology, Montefiore Hospital, N. Y. City

The hypothesis has been proposed that blood fluidity is preserved *in vivo* largely by cellular clearance of clotting intermediates (1). Data are reported which suggest that blood thromboplastin is removed from circulation by reticulo-endothelial cells(2). In the present study evidence is presented indicating that coagulation intermediate product I, a precursor of blood thromboplastin(3), disappears from blood of intact rabbits more rapidly than could be explained by action of circulating anticoagulants.

* Study supported by grants from Nat. Heart Inst., NIH.

Materials and methods. Experimental animals were male New Zealand rabbits weighing about 3 kg. Blood for preparation of product I was collected into uncoated glass tubes from central artery of ear. Plasma was obtained from blood collected into 1/10th volume of 3.8% sodium citrate, and aliquots were adsorbed with $Al(OH)_3$ as described by Pool and Robinson(4). Serum was prepared from blood incubated 1 hour after clotting at 37°C. Crude "cephalin" was made from human brain by method of Bell and Alton(5). Product I was prepared by mixing equal volumes of 0.025 M $CaCl_2$, undiluted serum, and

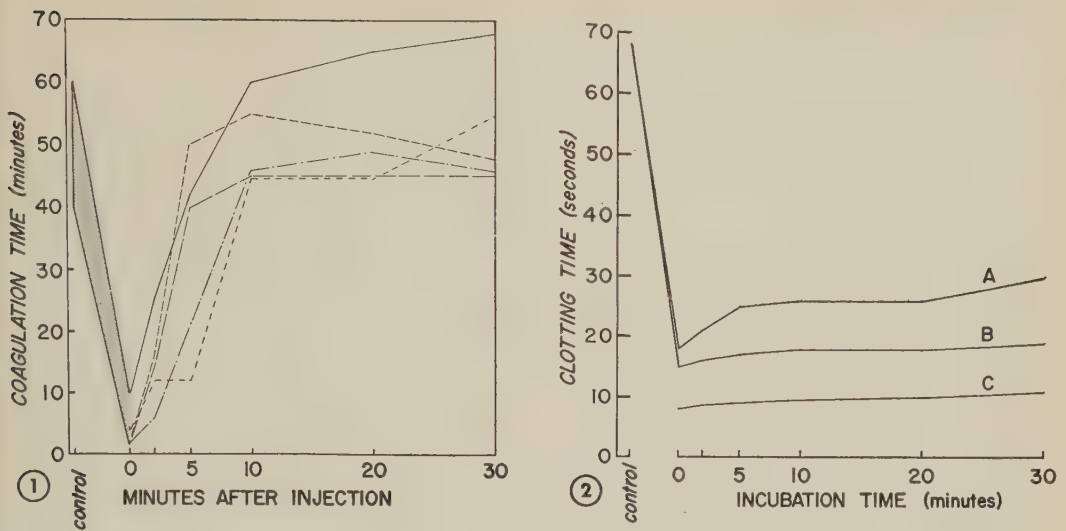


FIG. 1. Plastic tube clotting times following intrav. product I.
FIG. 2. *In vitro* inactivation of product I. (A) Diluted with 9 vol of plasma. (B) Diluted with 4 vol of plasma. (C) Undiluted. Control is recalcified clotting time plasma with optimal cephalin.

undiluted adsorbed plasma. This mixture was incubated at 37°C until full activity had developed and stabilized for at least 5 minutes. Product I activity was estimated by adding 0.1 cc of mixture to 0.1 cc of 0.025 M $CaCl_2$. To this was then added 0.1 cc of unadsorbed substrate plasma enriched with optimal concentration of cephalin, and clotting time determined. Optimal product I activity developed in about 10-15 minutes, causing substrate plasma to clot in 7-10 seconds. Times obtained with cephalin-free substrate plasma were 40 seconds or longer. Product I generating mixture clotted in about 7-10 minutes, and this clot was removed. Plastic tube clotting times were accomplished on blood drawn from right ventricle catheterized *via* right jugular vein as previously described(6). The effect of intravenous product I on coagulation time of rabbits was determined as follows: 20 cc of product I were given into marginal vein of left ear in about one minute. Clotting times were obtained before infusion, during administration of final few cc, and at intervals after completion of injection as indicated below. Additional blood was obtained through tubing for further clotting studies prior to injection and upon completion of experiment. *In vitro* inactivation of product I was estimated by diluting this reagent with various volumes of untreated plasma as indicated below. These mixtures were incubated at 37°C and aliquots repeatedly tested for product I activity as described above.

Results. The effect of intravenous product I on plastic tube clotting times in rabbits is shown in Fig. 1. There is striking acceleration of clotting during infusion, but this "hypercoagulable" state is rapidly reversed. Within 5-10 minutes after completion of infusion, the original clotting time is almost completely restored. Rapid administration of these large volumes of highly potent coagulant failed to cause detectable changes in prothrombin time, fibrinogen or thromboplastin generation, and reduction of platelets was modest: no *in vivo* defibrination was evident (Table I).

In vitro dilution of product I with plasma,

TABLE I. Coagulation Changes Following Product I Infusion (Mean Values).

	Before	After
Prothrombin (Quick) (sec.)	8.3	8.8
" (Ware & Stragnell) (sec.)	25	27
Fibrinogen (mg/100 cc)	240	225
Thromboplastin generation test (substrate time in sec. at 4 min. incubation)	8	8

comparable to that achieved *in vivo,* was followed by considerably less product I inactivation (Fig. 2). Loss of product I activity was slow, and even after 30 minutes considerable activity remained. These findings are in conformity with relative stability of product I reported by others(7).

Discussion. Formation of blood thromboplastin is evidently a multi-step reaction. Although the term "product I" is not entirely apt, and its nature is poorly defined, this intermediate can be partially characterized. Its formation depends upon previous activation of a plasma "contact factor" (Hageman factor) and presence of at least antihemophilic factor, Stuart factor, plasma thromboplastin component and Ca^{++}(7). Product I subsequently reacts with phosphatide and factor V to form blood thromboplastin(8). Unpublished studies by Dr. H. Horowitz of this laboratory indicate that reactions leading to formation of product I are slow, but that product I reacts with phosphatide and factor V almost instantaneously to form blood thromboplastin. The slow *in vitro* inactivation of product I here demonstrated is inadequate to account for the rapid restoration of plastic tube clotting time and it may be presumed that its removal from circulation of experimental animals represents a process involving clearance during passage through various organs. Although it appears unlikely that product I rapidly equilibrates with the entire extracellular fluid space, even this degree of dilution would not account for the loss of *in vivo* activity. The present data are not sufficient to suggest which cells or organs are responsible for product I clearance. In previous studies(1) intravenous administration of product I was not followed by increase in circulating anticoagulants. It therefore seems likely that product I is removed rather than neutralized. Rapid removal of product I from circulating blood appears an important component of hemostatic homeostasis, since its prolonged presence would lead to generalized clotting.

Summary. Removal of blood coagulation product I was studied in rabbits. Intravenous injection of this reagent markedly shortened plastic tube clotting time, but restoration to normal occurred in a few minutes. There was no evidence of defibrination. Mixed with plasma *in vitro,* product I was inactivated slowly. It is suggested that product I is removed from the circulation as blood passes through various organs.

1. Spaet, T. H., Kropatkin, M., *Am. J. Physiol.,* 1958, v195, 77.

2. Spaet, T. H., Horowitz, H. I., Zucker-Franklin, D. *J. Clin. Invest.,* 1960, v39, 1029.

3. Bergsagel, D. E., Hougie, C., *Brit. J. Haematol.,* 1956, v2, 113.

4. Pool, J., Robinson, J., *ibid.,* 1959, v5, 17.

5. Bell, W. N., Alton, H. G., *Nature,* 1954, v174, 880.

6. Spaet, T. H., Cintron, J., Krcpatkin, M., *J. Lab. & Clin. Med.,* 1959, v54, 467.

7. Fisch, U., Duckert, F., *Thromb. Diath. Haem.,* 1959, v3, 98.

8. Hougie, C., *J. Lab. and Clin. Med.,* 1957, v50, 61.

Received May 17, 1960. P.S.E.B.M., 1960, v104.

Experimental Epilepsy in the Mouse.* (25889)

LENORE M. KOPELOFF (Introduced by Warren M. Sperry)
Dept. of Bacteriology, N. Y. State Psychiatric Inst., N. Y. City

Previous studies demonstrated that a chronic epileptic state may be induced in the monkey (*Macaca mulatta*) by topical or intracerebral application of alumina cream to precentral motor cortex(1,2). Recently, certain pure metals implanted as pellets(3) or powders have also shown epileptogenic properties. Features of monkey epilepsy include spontaneous and induced clinical seizures, lowered thresholds to convulsant drugs(4,5), characteristic electroencephalographic abnor-

* Aided in part by Grant from NINDB, USPHS.

malities and associated focal cerebral lesion at site of application of inciting agent. The present study is concerned with production of a chronic convulsive state in the mouse.

Materials and methods. CF #1 female mice (Carworth Farms) were housed in groups of 4 to 6 in 6 x 10 plastic cages, and maintained on stock diet. At 7-8 weeks of age test material was implanted into brain (scalp incision, light ether) either by stylet through unbeveled tip of 1 mm 23-gauge hypodermic needle or by injection of 0.01-0.02 ml with similar 26-gauge needle. In most instances brains were prepared unilaterally in midsection of cerebrum at 2 sites approximately 3-5 mm from midline. Additional untreated mice were included as controls. Cobalt powder 200 mesh, nickel 325 mesh, antimony 140 mesh were sterilized several hours at 124°C in atmosphere of nitrogen to minimize oxidation. In addition, cobalt was autoclaved for comparative testing. Talcum powder USP, Falba† and aluminum oxide powder were sterilized in autoclave. Falba was emulsified with 2 volumes of saline for injection. Alumina cream was prepared as previously described(2). Post-treatment recovery in most mice was uneventful. Weekly observations were made during cage-cleaning and at random intervals for occurrence of "spontaneous" clinical seizures or those induced by routine handling. Challenge injections of pentamethylenetetrazole (Metrazol)(6) and semicarbazide(7) were selected at dosage levels below the range likely to elicit seizures in normal mice during relatively long experimental period (30 mg/kg subcut. Metrazol 0.3%; 50 mg/kg i.p. semicarbazide HCl 0.5%). Metrazol tests were performed initially at varying intervals after implantation and repeated at approximate 3-week intervals thereafter for 8-10 months. Semicarbazide tests were included toward latter part of experimental period. Control unoperated mice were tested as in treated series. Responses were graded positive when one or more generalized convulsions followed challenge or when only clonic movements of face and forelimbs were noted. Reactions following Metrazol usually occurred 5 to 17 minutes after injection, during 60-minute observation period. Onset of convulsions following semicarbazide appeared 24 to 196 minutes after injection, averaging 73 minutes, during 240-minute observation period. When a series of convulsions followed Metrazol or semicarbazide, amobarbital sodium was administered (50 mg/kg i.p.). Seizures following semicarbazide were further controlled by pyridoxine HCl (50 mg/kg i.p.), which was also administered to negative reactors at end of test period. Procedures of Toman(8) for "psychomotor" seizure test, and of Toman, Swinyard and Goodman(9) for submaximal seizure test were adapted by using shock levels below CD_{50} convulsive threshold for control groups. Rectangular pulses of 1 millisecond duration were delivered through saline-wick corneal electrodes at current values of 5.4-6.4 mA, at frequency of 6/second for 3 seconds for psychomotor test, and 9.5-12.5 mA at frequency of 100/second for 0.33 second for submaximal seizure test (Grass stimulator with oscilloscope). Tests were repeated at intervals of 1 day to 1 week until 2 trials at above mA values were obtained for each method.

Results. Cobalt. Preliminary findings indicated that cobalt powder implanted intracerebrally was an effective epileptogenic agent (occurrence of occasional spontaneous seizures and lowered threshold to Metrazol). To determine onset and duration of susceptibility to Metrazol, 73 mice prepared in either left or right cerebral hemisphere were divided into 7 comparable groups (9-13 mice) and challenged at various intervals. For control, 14 mice were prepared similarly with talcum powder, 12 with Falba and 38 left untreated. Following cobalt implantation (Table I) a lowered convulsive threshold to Metrazol became prominent by second week. At one week only 1 of 10 reacted positively. Between 2 and 10 weeks percentage of positive reactors varied from 40 to 92.3%, with highest initial susceptibility at 4 weeks. Retesting was carried out at 3-week intervals in most instances through 19th week. By this time deaths had

† Pfaltz and Bauer, Inc., N. Y.

TABLE I. Metrazol-Induced Seizures in Mice Following Unilateral Intracerebral Implantation of Cobalt.

Metrazol, 30 mg/kg subcut.			
Initial test		Subsequent tests*	
Wk after implantation	No. pos. / No. tested	Wk after implantation	No. pos. / No. tested
1	1/10	4	4/7
2	4/10	7	16/42
3	5/9	10	17/55
4	12/13	13	23/60
5	4/10	16	22/59
6	7/11	19	16/42
10	6/10	39	14/35
Total	39/73		112/300
Positive	53.4%		37.3%

* 3-5 wk after initial test and at succeeding 3-wk intervals. Controls—Initial test: untreated 0/38, talc 0/14, Falba 0/12. Subsequent tests: untreated 2/324, talc 0/99, Falba 0/91. Additional mice prepared as above with autoclaved cobalt, tested at 5 wk: 5/8 (62.5%) positive.

occurred in 14 mice of the cobalt series (19.2%), 2 of talc-prepared mice (14.3%), none in Falba group (0%) and 2 among untreated (5.4%). Weight increases among surviving animals showed no significant group differences. At 39th week approximately one-half the cobalt group was still alive and susceptible to Metrazol challenge at 40% level. This had remained relatively constant for the group, although mice as individuals did not react consistently on repeated testing. Reactions among controls were essentially negative.

Semicarbazide challenge was postponed until 22-38 weeks after cerebral preparation, when 2 tests were performed at 3-week intervals. No seizures were observed among control groups (11 talc, 12 Falba, 29 untreated). In 52 mice of cobalt series, there were 67 positive reactions to semicarbazide in 104 trials (64.4%). In the same mice, total positive reactions to Metrazol, including 2 further tests after semicarbazide, occurred 191 times in 469 trials (40.7%). When cobalt-treated mice were arranged according to individual scores to Metrazol challenge into: (a) those that were positive under one-third of times tested (24 mice) and (b) those positive over one-third of times tested (28 mice), positive reactions to semicarbazide challenge for the groups were: (a) 22/48 or 45.8% and (b) 45/56 or 80.4%.

Electroshock tests were performed following final Metrazol challenge in mice described above, the series including 41 cobalt-treated and 49 control mice (11 talc, 11 Falba, 27 untreated). In the psychomotor test, symptoms of minor seizures similar to those described by Toman, as well as "submaximal" seizures, were observed in 22 cobalt mice, and minor seizures alone in 11 controls. The difference between cobalt-treated and control group was significant ($p<.01$), and thus suggests a lowered psychomotor seizure threshold in cobalt-treated series.

When submaximal seizure test was applied to mice of the same groups, the reactions observed presented features characteristic of submaximal seizures in 8 of 41 cobalt-treated animals and in 1 of 49 controls. The difference between groups was significant ($p<.02$). This test also elicited reactions characteristic of minor seizures in 11 cobalt-treated and 3 controls, all but one of which (cobalt) failed to show submaximal seizures. This difference between groups was also significant ($p = .02$). The findings indicate therefore that cobalt treatment had lowered the threshold for submaximal and minor responses.

No routine attempt was made to elicit seizures by stressful prodding (as in experimental monkey epilepsy), to avoid possible effects on challenge with the convulsant drugs. "Spontaneous" seizures were distributed over entire test period and represented a minor proportion of all recorded convulsive reactions (58 spontaneous). No similar seizures were seen among control groups.

Nickel. Twenty-one mice received cerebral implantations of nickel powder either unilaterally or bilaterally. Few spontaneous clinical seizures were observed. Initial Metrazol challenge at 3 weeks proved positive in 5 of 12 prepared unilaterally (41.7%) and in 2 of 9 prepared bilaterally (22.2%). Subsequent retesting at 6-9, 12-19, 20-32, and 39-42 weeks (cumulative results) yielded positive group averages varying from 33.3 to 38.9% (unilateral) and 22.2 to 38.9% (bilateral). In 2 trials with semicarbazide 8 of

TABLE II. Production of Epilepsy in Mice by Intracerebral Implantation of Cobalt and Nickel.

Treatment	Metrazol,* 30 mg/kg subcut.			Semicarbazide,† 50 mg/kg i.p.		
	No. of mice	% mice positive	% tests positive	No. of mice	% mice positive	% tests positive
Cobalt	73	86.3	42.7	52	75.0	64.4
Nickel	21	85.7	33.8	14	57.1	42.9
Falba	21	0	0	16	0	0
Talc	25	0	0	24	0	0
Untreated	38	4.9	.6	30	0	0

Bilateral preparations: Nickel 9, Falba 9, talc 11.

* 1 to 10 trials over 10 mo period. † 2 trials on survivors 6-8 mo after preparation.

14 mice (4/7 unilateral, 4/7 bilateral) reacted positively (57.1%). Totals are included in Table II.

Antimony. This metal proved too toxic when applied to the brain as used for cobalt and nickel. Most mice so treated died within a few days to a week, although occasional survivors showed lowered convulsive threshold to Metrazol challenge. To prepare mice, therefore, antimony powder was mixed with talc (1:3, 1:7 or 1:15 by weight) before cerebral implantation. Of 11 mice so treated, 5 were positive to Metrazol on at least one trial. Five survivors at 38-44 weeks tested negative to semicarbazide.

Alumina cream was of especial interest because of its high degree of effectiveness in inducing epilepsy in the monkey. Numerous attempts to adapt its use to the mouse, employing 1 to 4 injection sites, unilaterally or bilaterally, as well as various dilutions of the suspended sediment, resulted in only occasional animals with spontaneous clinical seizures or lowered convulsive threshold to Metrazol. Implantation of aluminum oxide powder yielded negative results.

Focal nature of reaction. Clonic seizures confined to one side of body were observed in 14 mice prepared with cobalt unilaterally. Of these, 5 occurred spontaneously and all were contralateral to the prepared cerebral site. Of those reactions which followed Metrazol activation, 8 of 9 were contralateral to the cerebral site.

Discussion. Recurrent convulsive seizures in mice have certain features in common with those previously noted in experimental epilepsy in monkeys. These include incubation period following cerebral implantation of inciting agent, contralateral focal reactions, generalized tonic-clonic seizures, and lowered thresholds to convulsant drugs. Criteria for epilepsy in the mouse have been confined in our report to clinical seizures induced by challenge with Metrazol, semicarbazide and electroshock, at levels below convulsive thresholds for normal animals. Our findings are consistent with the knowledge that while the epileptogenic lesion (man) itself is chronic, clinical seizure discharges may be intermittent.

Use of the mouse as chronic experimental epileptic animal should prove of value in neurophysiologic, biochemical, pathologic and other studies, including screening and evaluation of anti-epileptics, as well as for investigations of effects of pharmacologic agents on the central nervous system.

Summary. Chronic epilepsy has been produced in the mouse by cerebral implantation of various powdered metals, notably cobalt and nickel. In addition to spontaneous focal and generalized seizures, epileptic status was evaluated by response to challenge injections of Metrazol and semicarbazide, as well as to electroshock.

Addendum. Cobalt implanted intracerebrally, in the manner described for mice, has recently proved highly epileptogenic in the rat as evidenced by lowered convulsive threshold to Metrazol.

1. Kopeloff, L. M., Barrera, S. E., Kopeloff, N., *Am. J. Psychiat.*, 1942, v98, 881.

2. Kopeloff, L. M., Chusid, J. G., Kopeloff, N., *A.M.A. Arch. Neurol. Psychiat.*, 1955, v74, 523.

3. Chusid, J. G., Kopeloff, L. M., *Fed. Proc.*, 1958, v17, 26.

4. Chusid, J. G., Kopeloff, L. M., Kopeloff, N., *J. Appl. Physiol.*, 1953, v6, 139.

5. Kopeloff, L. M., Chusid, J. G., Kopeloff, N.,

Proc. Soc. Exp. Biol. and Med., 1956, v92, 132.

6. Everett, G. M., Richards, R. K., *J. Pharm. Exp. Therap.*, 1944, v81, 402.

7. Jenney, E. H., Smith, R. P., Pfeiffer, C. C., *Fed. Proc.*, 1953, v12, 333.

8. Toman, J. E. P., *Neurology*, 1951, v1, 444.

9. Toman, J. E. P., Swinyard, E. A., Goodman, L. S., *J. Neurophysiol.*, 1946, v9, 231.

Received May 17, 1960. P.S.E.B.M., 1960, v104.

Effects of Administration of L-Thyroxin on Liver N-Demethylating Activity in Normal and Morphine-Treated Rats. (25890)

Joseph Cochin and Louis Sokoloff (Introduced by N. B. Eddy)

Lab. of Chemistry, Nat. Inst. of Arthritis and Metabolic Diseases and Lab. of Clinical Science Nat. Inst. of Mental Health, Bethesda, Md.

Chronic administration of morphine to rats causes a striking diminution in ability of liver microsomal-supernatant preparations from these rats to N-demethylate narcotic drug substrates(1,2). After abrupt withdrawal of drug from rats treated with morphine chronically, complete recovery of this enzyme activity occurs within 12 days(1,3). Recently L-thyroxin stimulated incorporation of amino acids into protein(4), suggesting that it accelerates protein synthesis. Since recovery of N-demethylase activity after withdrawal of narcotic drugs may represent enzyme resynthesis, it was of interest to study the effects of thyroxin on this process.

Methods. Forty-four NIH male black rats, each weighing 200-300 g, were divided into 4 groups so matched that average weight of each group was the same. The 4 groups were treated as follows. For 30 days, Group M received intraperitoneal injections of morphine sulfate in gradually increasing doses from 20 mg/kg to 100 mg/kg twice daily. Group MTh received morphine in same amounts and on same schedule as Group M and, in addition, a daily intraperitoneal injection of 90 μg of L-thyroxin in 0.5 ml of 0.01 N NaOH for 7 days prior to abrupt withdrawal of morphine and throughout period of withdrawal until day of sacrifice. Group Th received only thyroxin in same amounts and on same schedule as group MTh. Animals in group C were untreated controls. At 19 hours, 96 hours, and 8 days after termination of morphine administration, animals from the 4 groups were sacrificed and their livers prepared for enzyme assay by method of Axelrod(5). Enzymatic activity was measured by estimating the amount of formaldehyde formed by the N-demethylation of morphine, as described previously(2).

Results of representative experiment are summarized in Fig. 1. Nineteen hours after withdrawal of morphine and 7 days after initiation of thyroxin treatment in groups receiving the hormone, the N-demethylation of morphine by liver preparations from animals in group M was 6.7% of untreated-control value, and the activity in preparations from animals in group MTh was about one-half that of group M ($p<.01$). Preparations from animals receiving L-thyroxin alone for 7 days did not differ in N-demethylase activity from untreated controls. By 96 hours after withdrawal (and after 10 days of thyroxin administration in thyroxin-treated animals) N-demethylating activity began to recover in both groups M and MTh, and no significant difference between them was observed ($p>.05$). Then, a marked depression of activity was observed in animals treated with thyroxin alone (group Th). Eight days after morphine withdrawal, enzymatic activity of liver preparations from rats in group MTh was still less than one-fourth of control preparations (Group C) and had not increased appreciably from values obtained previously. On the other hand, N-demethylase activity for animals in Group M showed at this time an almost complete return to normal. Administration of thyroxin for 14 days had depressed

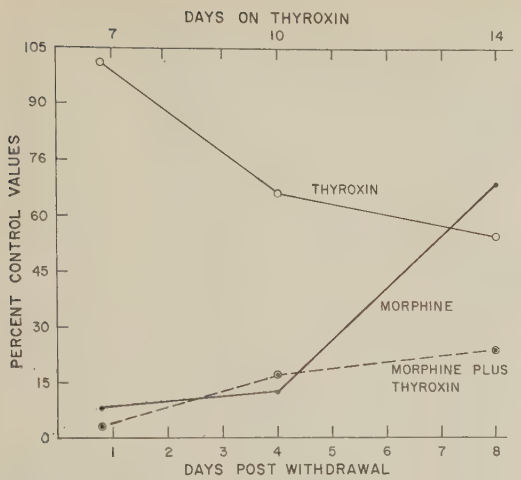


FIG. 1. Effects of administration of thyroxin on recovery of N-demethylating activity after morphine withdrawal.

liver N-demethylase in Group Th even more than previously.

To examine further the effects of chronic administration of thyroxin alone on *in vitro* N-demethylation of morphine, a group of male NIH black rats was given 90 μg of L-thyroxin daily to 29 days before liver enzyme activity was assayed. Table I compares enzyme activity after this treatment with that in untreated control rats of the same age and weight. Again, no effects of thyroxin were observed after only 7 days of administration, but after 10 days a distinct lowering of N-demethylase activity was observed. By 14 days enzyme activity was reduced to approximately half that found in control animals, and it remained at about this level until the end of 29-day period. Thus, thyroxin alone depresses ability of rat-liver-microsome preparations to N-demethylate morphine. This depression reaches a maximum in 10-14 days and remains at approximately the same level for at least an additional 2 weeks.

TABLE I. Effect of *In Vivo* Administration of L-thyroxin on *In Vitro* N-demethylation of Morphine.

Days on thyroxin	Activity (% of control)*†
7	100.9
10	65.7
14	54.4
21	43.0
29	57.7

* Each value represents mean of 7-10 thyroxin-treated animals compared to mean of 4 control animals sacrificed on same day.

† Enzymatic activity was measured by estimating amount of formaldehyde formed by N-demethylation of morphine hydrochloride.

These results indicate that thyroxin administration prior to and during withdrawal neither prevents morphine-induced diminution of N-demethylase activity nor increases its rate of recovery after withdrawal of morphine; instead, it causes a significant decrease in activity beyond that seen with morphine alone early in withdrawal, and a significant delay in recovery of enzyme activity. Under our conditions, administration of thyroxin during withdrawal period seemed to prevent recovery almost completely.

The significant difference between Groups M and MTh during early withdrawal, obliteration of this difference during middle period of withdrawal, and, finally, the markedly retarded recovery of demethylase activity in Group MTh during period when virtually complete recovery in Group M was observed are puzzling but reproducible phenomena. They may indicate a tendency for thyroxin to speed recovery obscured by another action simulating or perhaps potentiating the action of morphine. The nature of the mechanisms underlying the effects on N-demethylase activity by morphine and thyroxin singly and together remains, however, unknown.

Summary. The effect of *in vivo* administration of L-thyroxin on *in vitro* N-demethylation of morphine by rat-liver enzyme preparations from normal and morphine-treated rats has been investigated. Thyroxin given 7 days to otherwise untreated control animals has no effect on N-demethylating activity, but after 10-14 days it significantly depresses this activity. Administration of thyroxin for 7 days prior to and during morphine withdrawal reduces further the enzyme activity already markedly depressed after chronic administration of morphine, and prevents almost completely recovery of activity which occurs some 8-10 days after morphine withdrawal.

We express appreciation to Elmer Dyson and Donald Shaw for invaluable technical assistance.

1. Axelrod, J., *Science*, 1956, v124, 263.

2. Cochin, J., Axelrod, J., *J. Pharmacol. Exp. Therap.*, 1959, v125, 105.

3. Cochin, J., Economon, S., *Fed. Proc.*, 1959, v18, 377.

4. Sokoloff, L., Kaufman, S., *Science*, 1959, v129, 569.

5. Axelrod, J., *J. Pharmacol. Exp. Therap.*, 1956, v117, 322.

Received May 23, 1960. P.S.E.B.M., 1960, v104.

Effect of Pre-Immunized Rat Bone Marrow on Lethally Irradiated Mice. (25891)

PAUL H. CHIN AND MYRON S. SILVERMAN*

Biological and Medical Science Division, U. S. Naval Radiological Defense Lab., San Francisco, Calif.

Post-irradiation protection of lethally irradiated mice by injection of heterologous (rat) marrow is associated with rapid repopulation of the host's depleted hematopoietic tissue by the transfused marrow cells(1,2). The treated animals initially recover from radiation induced injury, but a significantly large percentage of deaths characteristically occur after third week of irradiation treatment(3,4). Although *direct immunological* evidence is still lacking, it is generally accepted that the pathogenesis of these "late deaths" is immunologic in origin. It is still uncertain whether the host or the transplanted graft initiate the immune reaction, nor is it known whether the pathological foundation of "late deaths" is circulating or fixed tissue antibodies. The experiments to be described were performed to compare time and incidence of deaths in lethally irradiated mice injected with bone marrow from normal rats and with bone marrow from rats immunized against tissues of the recipient strain of mice. On the basis of a graft *vs.* host reaction, it would be expected that both time and incidence of death would be accentuated following injection of pre-immunized rat marrow, resulting from direct restimulation of transplanted cells by the host's antigens. In contrast, if a host *vs.* graft reaction occurred or if colonization of immunologically active donor cells was unsuccessful no difference would result between groups receiving normal or pre-immunized marrow. Our results favor the latter conclusion.

Materials and methods. Sprague-Dawley albino rats, 12 to 18 weeks of age, were immunized against spleen and bone marrow suspensions prepared from normal adult LAF_1 ($(C57LxA)F_1$) mice. The mice were sacrificed by cervical dislocation. Spleens were excised, pooled, minced with fine scissors, then pressed through a 60x60 mesh stainless steel wire gauze. The cells were suspended and washed twice with chilled Tyrode's solution. Large clumps were removed by filtration through surgical gauze, and a cell count made in a hemacytometer. The desired concentration of cells was prepared by addition of Tyrode's solution. Mouse marrow cells were obtained by aspiration from the femurs and a suspension was prepared in Tyrode's solution. Three separate experiments were performed differing only in number of immunizing injections administered to the rat donors and in time after immunization at which the rats were sacrificed for collection of bone marrow and spleen cells. In the initial experiment, 2 intravenous injections of $1x10^8$ nucleated spleen cells were given 9 days apart followed by sacrifice 3 weeks after last injection. Serologic test of pooled sera from 10 rats showed a hemagglutinating titer of 1:512 against washed LAF_1 mouse rbc. In the second experiment, rats were given 2 intravenous injections of $1x10^8$ spleen cells a week apart followed by a third injection 2 weeks afterwards. These donor animals were sacrificed

* Opinions or assertions contained herein are those of authors and are not to be construed as official or reflecting views of Navy Department or of Naval Service at large.

TABLE I. Mortality and Mean Survival Time of LAF_1 Mice Receiving 870 r Whole Body X-Irradiation Followed by Injection of Immunized or Non-Immunized Rat Cell Preparations.

Exp.	Treatment of irradiated LAF_1 mice	No. of mice per group	% mortality (days) 30	60	90	120	150	Mean survival time of decedents (days) 30	60
21	1×10^8 imm. RBM*	25	56	80	80	80	80	18.9	26.5
	" non-imm. RBM	25	32	60	80	80	80	14.4	26.5
44	7×10^7 imm. RBM†	41	56	80	95	98	98	17.8	23.5
	" non-imm. RBM	39	46	74	74	80	87	21.7	27.2
48	" imm. RBM‡	35	29	69	80	86	89	14.9	27.6
	" non-imm. RBM	26	31	69	85	89	96	12.9	27.2
44 sp.	7×10^7 non-imm. RBM + 1×10^7 imm. rat spleen cells‡	18	44	72	72	78	78	20.2	27.9
	7×10^7 non-imm. RBM + 1×10^7 non-imm. rat spleen cells	28	36	64	71	75	75	19.3	27.8
48 sp.	7×10^7 non-imm. RBM + 5×10^7 imm. rat spleen cells‡	15	100					8.1	
	7×10^7 non-imm. RBM + 5×10^7 non-imm. rat spleen cells	15	100					12.0	

* Donor rats given 2 intrav. inj. of LAF_1 spleen cells.
† " " " 3 " " " " " " .
‡ " " " 4 " " " combined LAF_1 spleen and bone marrow cells.

9 days after the last injection. The hemagglutinating titer of the pooled sera was 1:512. Four weekly intravenous injections of combined mouse spleen and bone marrow cells were administered to donor rats in the third experiment. Each animal received a total of $1x10^8$ cells per injection. Eight days following last injection, the animals were sacrificed. The pooled sera from this group of rats similarly agglutinated LAF_1 mouse rbc with a titer of 1:512. Rat bone marrow for injection of irradiated mice was collected by the method described by Fishler, *et al.*(5) and was suspended in chilled Tyrode's solution containing 25,000 units of crystalline penicillin G per ml. A volume of 0.2 ml was injected intravenously per mouse. Spleen cell suspensions were prepared as described for preparation of mouse spleen cells. Recipient LAF_1 mice, 12 to 18 weeks of age, were exposed to a single dose of 870 r whole body X-irradiation. The X-ray source was a 250 kvp Westinghouse Therapy unit. Radiation factors were 15 ma; filter 0.5 mm Cu plus 1.0 mm Al; skin to target distance 40 inches, dose rate approximately 25 r/min measured in air. The mice were injected intravenously with the required rat cell preparation within 4 hours after irradiation. An equal number of animals was selected at random from each group and housed together. A maximum of 10 animals per cage was allowed.

Results. The results obtained from the 3 different experiments are presented in Table I. The majority of irradiated mice were dead by the 60th postirradiation day regardless of immunological status of the rat cells used for injection. A comparison between number of deaths of recipients of immunized or normal rat marrow by the chi-square method showed no significant differences between the 2 groups of animals in each experiment when tested at each 30-day interval. ($P>.1$ in all cases). A similar analysis revealed no significant increase in number of deaths when $1x10^7$ pre-immunized rat spleen cells were injected into recipient mice as compared to recipients of an equivalent amount of nonimmunized rat spleen cells.

Since the greatest number of "late deaths" occurred within the initial 60-day period, it would be expected that any enhancing effect on mortality by pre-immunized rat marrow or spleen cells would be manifested within this interval. This is based on the assumption that a graft *vs.* host immunological reaction occurs and that pre-immunization against the recipient would therefore result in an accelerated secondary response upon injection into the recipient animals. A comparison of mean

survival times at the 30- and 60-day period was made by use of the "t" test. No significant difference in mean survival time was found within each bone marrow experiment, either at the 30- or 60-day period. ($P>.1$). In 2 of these experiments mean 30-day survival time of the animals injected with pre-immunized marrow was slightly longer than that of its respective control group.

A similar comparison of mean survival time showed no significant difference between recipients of non-immune bone marrow and either $1x10^7$ normal or pre-immunized spleen cells Exp. 44 sp). It is also of interest to note that mean survival time of these animals is comparable to that of animals receiving rat bone marrow alone in the same experiment (Exp. 44). When the concentration of spleen cells injected was increased to $5x10^7$ cells, a highly significant decrease in mean survival time was found for animals receiving pre-immunized cells when compared with recipients of an equivalent amount of normal spleen.

Discussion. These results suggest that following injection of heterologous bone marrow either proliferation of the comparatively small number of immunologically active cells may be slow or proliferation of these cells may not even occur. Either of these events could then prevent the reaction of the graft against the host. These findings are in contrast to those of Cosgrove *et al.*(6) who reported enhancement of foreign bone marrow reaction following injection of pre-sensitized parental marrow into F_1 recipient mice. The basis for this variability in results may be due to the greater difference in genetic relationship between donor and recipient in the present experiments. Shekarchi *et al.*(7) have reported that acceptance of foreign grafts is directly related to genetic difference between graft and host. A higher dose of irradiation was required to impair the immune mechanism of the host against a more distantly related antigen. Since the same X-ray dose is usually employed in post-irradiation protection studies with homologous and heterologous bone marrow, a difference in initial acceptance of these grafts may result. The large amount of rat marrow cells required for "protection" as contrasted to the small amount of homologous cells needed (approximately 14-1 on wet weight basis) may be further evidence of a difference in primary acceptance of these cells and might be related to an increase in the statistical probability that a sufficient number of injected rat cells will survive to provide "protection" against acute radiation injury. In view of the small amount of lymphopoietic elements present in bone marrow, the initial destruction, by immunologic or other mechanisms of a large proportion of the injected rat cells may result in the selective colonization and subsequent proliferation of myelopoietic and erythropoietic elements only. The relative increase in circulating granulocytes(2,8) observed in rat bone marrow protected animals may be a reflection of such a selection. Further restriction on growth and proliferation of immunologically active homologous cells was suggested by the work of Boyse(9) who reported that antibody forming cells are particularly susceptible to immune attack and may therefore be eliminated sooner than other types of cells.

The inability of a supplementary injection of $1x10^7$ normal or preimmunized rat spleen cells to alter significantly mean survival time of bone marrow treated animals when compared with animals receiving bone marrow alone is further evidence for lack of extensive proliferation of heterologous immunologically active cells. In this connection, it was shown (8) that injection of bone marrow or $1x10^7$ spleen cells from rats immunized against sheep red blood cells into lethally irradiated mice did not result in transfer of antibody synthesis. However, injection of $5x10^7$ or a greater amount of spleen cells from the same immunized donors successfully transferred production of rat antibody against sheep red blood cells to the irradiated recipients. Coincidentally, a highly significant difference was observed in the present experiment between animals receiving $5x10^7$ pre-immunized rat spleen cells and $5x10^7$ normal spleen cell. In view of the short survival time of these animals, it does not appear that extensive pro-

liferation of injected cells was necessary for expression of its lethal effect.

Although growth and proliferation of heterologous (rat) myeloid cells have been successfully demonstrated in lethally irradiated mice injected with rat bone marrow, colonization by immunologically active lymphoid cells has not been conclusively shown. Gengozian *et al.*(10) and Zaalberg *et al.*(11) have reported the repopulation of lymphoid tissue of rat-mouse chimeras by donor cells. However, cell types and ability of these cells to function immunologically was not tested. Conflicting reports have also appeared regarding presence (12) or absence(13) of circulating rat type gamma globulin in rat-mouse chimeras. In view of the lack of conclusive evidence bearing on proliferation of immunologically active heterologous (rat) lymphoid cells, serious reservation must be placed on a graft *vs.* host reaction as an explanation for "late deaths" *in rat-mouse chimeras.*

Summary. A comparison was made of time and incidence of death between lethally irradiated mice injected with non-immune rat bone marrow and those injected with marrow from rats pre-immunized against recipient strain of mice. Statistical analysis of data showed no significant differences between the 2 groups of animals. Similarly, a lack of significant difference was observed with marrow-treated mice given a supplementary injection of moderate amounts of either non-immune or pre-immunized rat spleen cells. The failure of sufficient numbers of immunologically active cells to colonize and proliferate is discussed as the most probable interpretation of the results.

1. Nowell, P. C., Cole, L. J., Habermeyer, J. G., Roan, P. L., *Cancer Res.,* 1956, v16, 258.

2. Makinodan, T., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 174.

3. Congdon, C. C., Lorenz, E., *Am. J. Physiol.,* 1954, v176, 297.

4. Cole, L. J., Habermeyer, J. G., Bond, V. P., *J. Nat. Cancer Inst.,* 1955, v16, 1.

5. Fishler, M. C., Cole, L. J., Bond, V. P., Milne, W. L., *Am. J. Physiol.,* 1954, v177, 236.

6. Cosgrove, G. E., Jr., Upton, A. C., Schwartz, E. E., Congdon, C. C., Makinodan, T., PROC. SOC. EXP. BIOL. AND MED., 1959, v100, 417.

7. Shekarchi, I. C., Makinodan, T., *ibid.,* 1959, v100, 414.

8. Chin, P. H., Silverman, M. S., *J. Immunol.,* in press.

9. Boyse, E. A., *Immunology,* 1959, v2, 170.

10. Gengozian, N., Urso, I. S., Congdon, C. C., Conger, A. D., Makinodan, T., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 714.

11. Zaalberg, B. O., van Bekkum, D. W., *Transplantation Bull.,* 1959, v6, 91.

12. Grabar, P., Courcon, J., Illbery, P. L. T., Loutit, J. F., Merrill, J. P., *Compt. rend. Acad. Sci.,* 1957, v245, 950.

13. Gengozian, N., *J. Immunol.,* 1959, v83, 173.

Received May 24, 1960. P.S.E.B.M., 1960, v104.

Histochemical Changes in Kidneys and in Salivary Glands of Rats with Experimental Hypertension. (25892)

F. GROSS AND R. HESS (Introduced by F. F. Yonkman)

Pharmaceutical Dept., CIBA Ltd., Basle, Switzerland and Inst. of Pathology, University of Basle

Experimental hypertension produced in the rat by overdosage with cortexone acetate (DCA) and salt leads to disappearance of renin from the kidneys and to diminution of glucose-6-phosphate dehydrogenase activity in the macula densa(4). Identical changes may be observed in the unclamped kidney of rats in which hypertension is provoked by clamping one renal artery(5). These observations led us to conclude that the unclamped kidney in animals with renal hypertension is subject to similar pathogenic influences as are the kidneys of animals overdosed with DCA and salt. The fact that glucose-6-phosphate dehydrogenase (G6PD) activity is demonstrable in other structures in addition to the macula densa, *e.g.,* the small and medium ducts of salivary glands, induced us to deter-

mine whether the activity of this enzyme in the salivary gland shows changes comparable with those seen in the kidney under identical experimental conditions.

Methods. Inbred male albino rats, initial weight 120-145 g, were used in groups of 3 or 4. They were fed commercial ("Nafag") pellets and had free access either to tap water or, in the DCA overdosage experiments, to 1% saline. Hypertension was produced either by subcutaneous injection of DCA at a dose of 50 mg/kg of a microcrystalline suspension given at weekly intervals, or by unilateral clamping of the renal artery with a silver ribbon clip. Systolic blood pressure was measured under light ether anesthesia using a tail plethysmograph(1). The effect of adrenal insufficiency was studied in bilaterally adrenalectomized rats maintained on a low sodium diet and which were sacrificed 7 days after operation. All the animals were killed by cervical dislocation and were bled. Blocks of tissue from the middle part of both kidneys and whole submaxillary glands were removed quickly and immediately frozen in CO_2 and stored in a cryostat at –20°. G6PD, di- and triphosphopyridine nucleotide diaphorase (DPN- TPN-diaphorase) activity was demonstrated histochemically in 8 μ tissue sections by a cobalt-formazan technic(2,3). Untreated and sham-operated animals maintained on tap water or saline served as controls.

Results. In control animals, moderately strong G6PD activity could be demonstrated histochemically in the macula densa cells of the renal cortex and in the excretory duct epithelium of both serous and mucous parts of the salivary gland(3) (Fig. 1,4). Variations in enzyme activity in these structures occurred in animals with experimental hypertension or adrenal deficiency.

Overdosage with DCA and salt. Concomitant with development of hypertension, a gradual decrease of G6PD activity occurs in the macula densa cells of the kidneys (Fig. 5) and the activity was greatly diminished after 4 weeks' treatment(4). When G6PD activity in macular cells was compared with activity in salivary duct epithelium, a simultaneous decrease could be demonstrated in both structures. In the salivary glands, decreased G6PD activity was accompanied by flattening of the epithelium and distension of the excretory ducts (Fig. 2). The latter was similar to the widening of the lumen of the cortical tubular system in the kidney which can be observed to follow DCA-saline overdosage of more than 2 weeks' duration. Decrease of G6PD activity was confined to the salivary duct system. The high activity of this enzyme in elements of the autonomic ner-

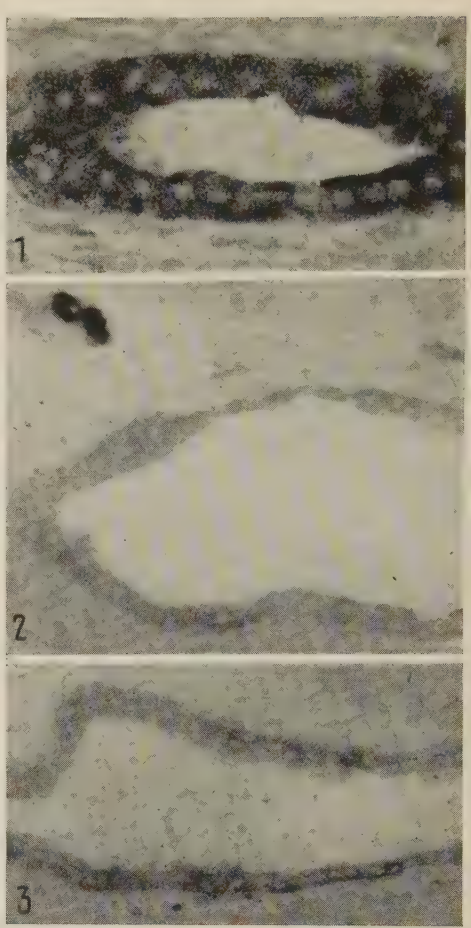


FIG. 1. Normal (high) activity of G6PD in excretory duct epithelium of rat submaxillary gland. 380 ×.

FIG. 2. DCA-NaCl treatment for 4 wk. Markedly decreased G6PD activity in ductal epithelium, in contrast to that in autonomic ganglion cells (upper left). 200 ×.

FIG. 3. Widened salivary duct, with epithelial cells displaying weak G6PD activity, from a rat bearing a Goldblatt clamp. 200 ×.

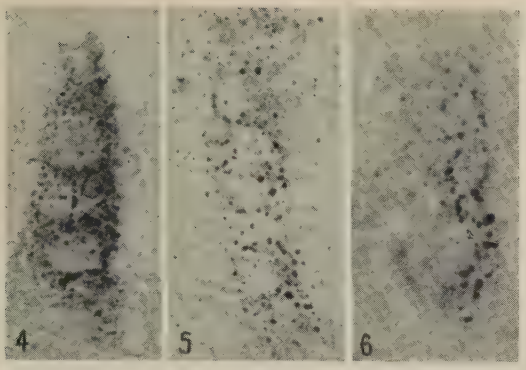


FIG. 4. Normal activity of G6PD in macula densa cells (untreated control animal). 1000 ×.

FIG. 5. Decreased G6PD activity in macula densa of a rat treated with DCA-NaCl for 2 wk. 1000 ×.

FIG. 6. Same animal as Fig. 3. Weak G6PD activity in macula densa cells of unclamped kidney. 1000 ×.

vous system within the gland was preserved (Fig. 2). TPN diaphorase activity in the excretory ducts was slightly diminished as compared with control animals. No change was noted in activity of DPN diaphorase.

Renal hypertension. Four to 5 weeks after unilateral clamping of the renal artery, G6PD activity in the salivary duct epithelium was depleted, the rats at this time having a raised blood pressure of 185-205 mm Hg (Fig. 3). Decreased enzymatic activity was accompanied by dilatation of the excretory ducts and closely resembled changes noted after DCA-NaCl overdosage for 3 weeks. As estimated by visual comparison, the decrease in G6PD activity in the salivary ducts was accompanied by a similar fall of activity in the macula densa which regularly occurred in the non-clamped kidney (Fig. 6). The clamped kidney, in contrast, showed increased G6PD activity in macular cells(5). No marked changes in diaphorase activities were observed.

Adrenal insufficiency. In rats showing signs of adrenal insufficiency, G6PD activity in the salivary duct epithelium was approximately equal to that seen in control animals. Confirming a previous observation(4), G6PD activity in the macula densa was shown to rise following adrenalectomy.

Discussion. The fact that in 2 different forms of experimental hypertension G6PD activity shows parallel changes in 2 organs with completely different functions demonstrates that variations hitherto observed only in the kidney also occur under similar conditions in other tissues. Although it is tempting to speculate that a humoral factor might be responsible for these parallel changes, no proof for such a hypothesis is available and the alternative explanation that a common underlying pathogenic mechanism is responsible for the alterations in various organs without interference of a humoral factor is equally probable. In the 2 forms of experimental hypertension, changes in sodium balance also occur and it is known that after a period of hypertension comparable to that after which these histochemical analyses were done, accumulation of sodium may be demonstrated in various tissues(6,7). On the other hand, the kidney as well as the salivary glands are secretory organs, eliminating salt and water, and similar changes in enzymatic activity in their duct systems may be the expression of attempts to adjust the function of these organs to special conditions. This interpretation agrees with the opinion expressed previously that in animals with unilateral clamping of the kidney, decrease in renin content and in G6PD activity in the untouched kidney represent efforts to counteract retention of sodium(8). The clamped kidney, however, cannot adapt its enzyme activity in the same way as the unclamped kidney. It is not possible to decide whether these changes in enzyme activity are directly involved in these functions or whether they are only an expression of diminution of activity in a primary energy yielding process on which some as yet unknown or unidentified mechanism for sodium transport depends.

Summary. In rats with experimental hypertension due either to overdosage with DCA and salt or to unilateral clamping of renal artery, activity of glucose-6-phosphate dehydrogenase (G6PD) was determined histochemically in the macula densa and simultaneously in ducts of submaxillary gland. In both kidneys of animals with DCA hypertension and in unclamped kidney of animals with renal hypertension, G6PD is diminished to a comparable degree, both in macula densa cells and

in the duct epithelium of submaxillary gland, whereas activity in the clamped kidney is higher than normal. Simultaneously there is a widening of ducts in the salivary gland comparable to that seen in cortical tubular system in the kidney. Adrenalectomy is followed by increased G6PD activity in the macula densa and a high normal activity in the submaxillary gland.

1. Byrom, F. B., Wilson, C., *J. Physiol.,* 1938, v93, 301.

2. Hess, R., Scarpelli, D. G., Pearse, A. G. E., *J. Biophys. Biochem. Cytol.,* 1958, v4, 753.

3. Scarpelli, D. G., Hess, R., Pearse, A. G. E., *J. Biophys. Biochem. Cytol.,* 1958, v4, 747.

4. Hess, R., Gross, F., *Am. J. Physiol.,* 1959, v197, 869.

5. Hess, R., Pearse, A. G. E., *J. Exp. Path.,* 1959, v40, 243.

6. Tobian, L., Binion, J. T., *J. Clin. Invest.,* 1954, v33, 1407.

7. Gross, F., Schmidt, H., *Arch. exp. Path. u. Pharmakol.,* 1958, v233, 311.

8. Gross, F., *Klin. Wschr.,* 1958, v36, 693.

Received May 24, 1960. P.S.E.B.M., 1960, v104.

Spontaneous Hydronephrosis in the Rat.* (25893)

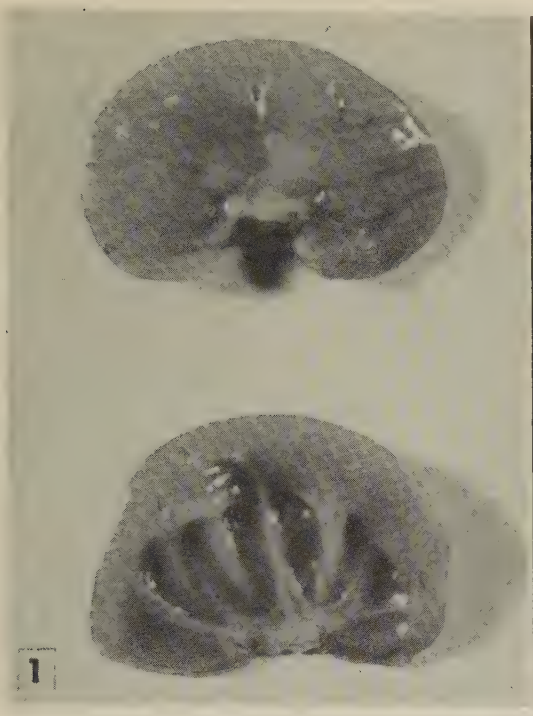
ALVIN L. SELLERS, SHELDON ROSENFELD AND NATHAN B. FRIEDMAN
Inst. for Medical Research, and Division of Laboratories, Cedars of Lebanon Hospital, Los Angeles

Investigators using the Slonaker-Addis albino rat have long been aware of the frequent occurrence of spontaneous hydronephrosis in this strain. Recent interest in experimental production of pyelonephritis in the rat stimulated us to study this phenomenon in more detail.

Animals used represent a widespread strain of albino rats. Slonaker brought originators of the colony to California in 1903 from the Neurological Dept., University of Chicago. From the same colony, at a later date, the Wistar Institute Colony was derived. Thomas Addis used these rats to start the Stanford University Colony in 1924. In 1948 Addis moved a portion of the colony to Cedars of Lebanon Hospital, where it has been referred to as the Slonaker, and more recently, the Slonaker-Addis strain. Since 1924, the colony has been inbred, and great care taken to prevent cross-breeding with other strains.

Methods and results. Presence of hydronephrosis was determined by simple inspection of sliced kidney. Hydronephrosis of all degrees was seen, but in the majority of instances resembled that shown in Fig. 1. Groups of male and female rats weighing 90-120 g, and 200-350 g were sacrificed to determine incidence of hydronephrosis in the colony as a function of age and sex. The results are shown in Table I. In all instances, hydronephrosis occurred in the right kidney. Absence of hydronephrosis in adult female rats was striking, and it was suggested that pregnancy might be responsible. A group of 50 pre-puberty female rats were allowed to mature. Twenty-five females were mated and survived a normal pregnancy. The other 25 were allowed to mature without becoming pregnant. There were no deaths in either group. All animals were sacrificed after achieving a weight in excess of 200 g. Hydronephrosis was not found. Thus, right hydronephrosis, found in approximately 45% of immature female rats, disappears as animals mature regardless of whether or not they have borne young. Hooded rats of Long-Evans strain, as well as albino rats of Wistar strain, were inspected for hydronephrosis, and none was found (Table I). *X-ray Technic.* Presence of hydronephrosis can be determined without sacrificing the animal by intravenous urography. The x-ray technic most suitable is as follows: 42 kilovolts, 1/60 sec., 300 milli-

* Aided by grants from Nat. Heart Inst., U.S.P.H.S. and Southern Calif. Chapter, Nat. Kidney Disease Fn.



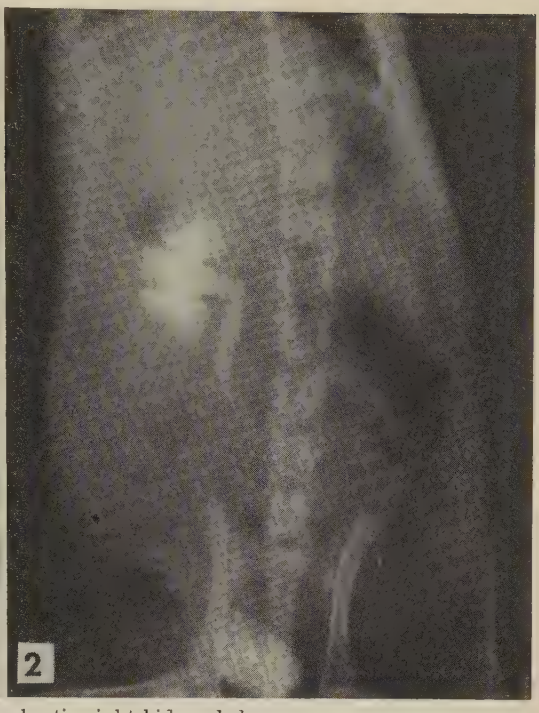


FIG. 1. Normal right kidney above, hydronephrotic right kidney below.
FIG. 2. X-ray exposure 10 min. after intrav. inj. of renografin®. Dye has already been excreted through left kidney.

amperes, non bucky screen, 38″ distance, using 8-10 inch cone. Rats were anesthetized with ether and injected intravenously with 0.5 ml of 60% renografin®/100 g body weight. A single film taken after 10 minutes reveals the presence of hydronephrosis. (Fig. 2).

Anatomical findings. It is apparent from inspection of intravenous urograms that hydronephrosis is associated with at least partial obstruction of the right ureter at junction of its upper and middle third in the male, and slightly higher in the female (Fig. 2). The right ureter crosses ventral to the ileolumbar artery and vein, then courses toward the bladder in close association with and just to the right of the inferior vena cava, and very near the midline. As the ureter enters the pelvis, it swings somewhat more laterally to enter the bladder (Fig. 3). The left ureter on the other hand is well lateral to midline structures throughout its course from kidney to bladder.

In the male, the right internal spermatic artery leaves the aorta distal to renal arteries and crosses ventral to the right ureter at the point where ureter crosses the ileolumbar vessels, thus the ureter lies between the ileolumbar vessels dorsally and the internal spermatic vessels ventrally (Fig. 3). It is invariably at this point that the right ureter is obstructed. It should be stressed that anatomical relationships described above exist whether hydronephrosis occurs or not. However, when hydronephrosis and hydroureter do occur, it is always by obstruction at the point indicated. No such crossing of ureter by the left internal spermatic artery occurs, since the left

TABLE I. Incidence of Spontaneous Right Hydronephrosis in the Rat.

Wt, g	Sex	No. animals	Hydronephrosis	%
	Slonaker-Addis strain			
90–120	♂	23	14	61
	♀	25	11	44
200–350	♂	26	12	46
	♀	26	0	0
	Long-Evans strain			
90–100	♂	14	0	0
	♀	11	0	0

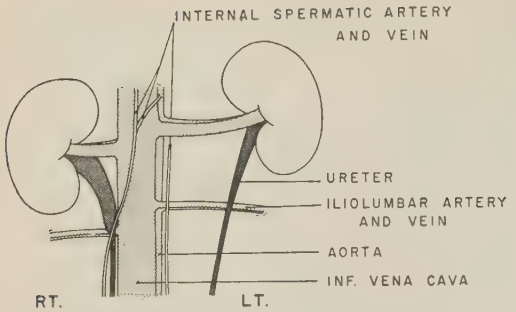


FIG. 3. Anatomic relationships of ureters in Slonaker-Addis albino rat. Note partial obstruction of right ureter as it lies between internal spermatic, and ileolumbar vessels.

ureter is lateral to this vessel, throughout its course.

In the female, the course of ureters is similar to that seen in the male; however, obstruction to the ureter occurs where it is crossed by the ovarian vessels.

In the Long-Evans strain, the anatomical relationship of the right ureter is similar to that of the left ureter. Both structures course from kidney to bladder well lateral to the internal spermatic arteries.

Bacteriological findings. Hydronephrotic right kidneys, and nonhydronephrotic left kidneys were removed aseptically. The organs were minced and cultured aerobically and anaerobically on blood agar plates. Aliquots of tissue were placed in brain-heart infusion broth and thioglycollate broth. Cultures were held 48 hours. Results are shown in Table II.

From a total of 42 hydronephrotic kidneys only 4 contained microorganisms. *Escherichia coli* was grown from 2 kidneys. In one of these the growth was heavy and in one, only a few organisms were found. A heavy growth of *Escherichia coli* was obtained from a single left kidney. One must conclude that the majority of hydronephrotic kidneys are sterile and that one is nearly as likely to culture organisms from the normal left kidney. Thus, it does not appear that the hydronephrotic lesion of the right kidney greatly predisposes this kidney to spontaneous infection regardless of length of time the lesion is present.

TABLE II. Results of Culture of Minced Hydronephrotic Rat Kidney and Normal Left Kidney.

No. animals	Wt, g		*E. coli*	Pneumococcus	Sterile
15	90–120	R. kidney	0	1	14
		L. "	1	1	13
27	200–300	R. "	2	1	24
		L. "	0	0	27

Aside from dilation of the renal pelvis, the hydronephrotic kidney is essentially normal. There is no significant abnormality of glomeruli or renal tubules and histology of renal vasculature is normal. There is no histological evidence of interstitial infiltration of pyelonephritis. Serial sections of the ureter taken from above, through and below site of obstruction fail to reveal any abnormality in structure of the ureter.

Discussion. A strain of inbred albino rats is described in which a striking incidence of spontaneous right hydronephrosis occurs. This abnormality is caused by partial obstruction of the ureter by either internal spermatic or ovarian arteries and related to the right ureter which lies in close relationship to midline inferior vena cava. Absence of hydronephrosis in adult female rats must in some way be related to altered anatomical relationships secondary to growth and does not depend on pregnancy or failure of young females with hydronephrosis to survive. Hydronephrosis did not predispose the right kidney to infection. Cultures revealed a low incidence of significant infection in either hydronephrotic right or normal left kidneys and histological sections of the hydronephrotic kidney did not reveal evidence of pyelonephritis. Aside from any interest that this abnormality may have for the anatomist, urologist, or geneticist, the strain may prove useful for study of experimental pyelonephritis.

Summary. 1) Spontaneous right hydronephrosis occurs in approximately half the animals in an inbred strain of Slonaker-Addis albino rats. The lesion disappears in female rats as they exceed 200 g weight. 2) The abnormality is produced by partial constriction of the right ureter by the ovarian or spermatic artery. 3) Hydronephrosis does

not appear to predispose the animals to spontaneous infection in the abnormal kidney.

The authors express their thanks to Drs. David Zion, Dept. of Radiology and to Maurice White, Dept. of Bacteriology, for aid in this study.

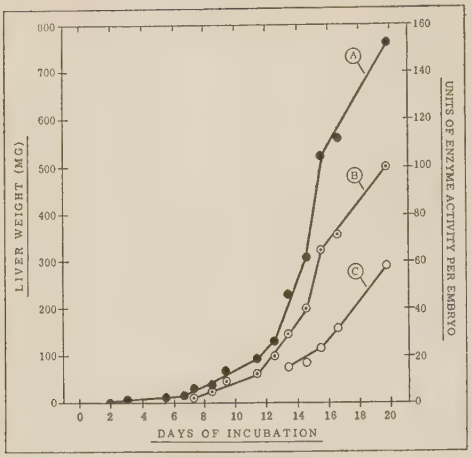
Received May 25, 1960. P.S.E.B.M., 1960, v104.

Hepatic Glucose Production in Developing Chicken Embryo. (25894)

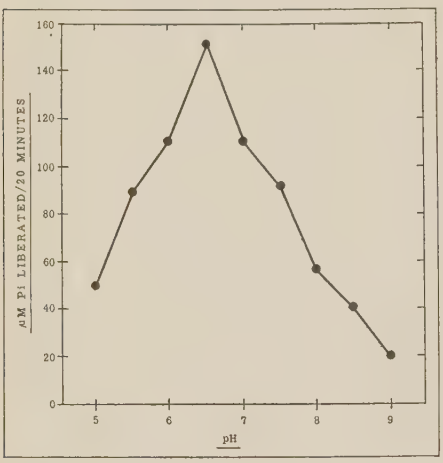
GRACE S. KILSHEIMER, DAVID R. WEBER AND JAMES ASHMORE
Dept. of Biochemistry, Indiana University Medical School, Indianapolis

Previous studies by Nemeth(1) and Weber and Cantero(2) have demonstrated an absence of glucose-6-phosphatase activity in fetal guinea pig and rat liver. This enzyme is also absent or of low activity in Von Gierke's disease and is believed to be the primary enzymatic defect which leads to glycogen storage and hypoglycemia characteristic of this disease(3). Since glucose-6-phosphatase activity is not found in fetal liver of 2 species examined the suggestion has been made that Von Gierke's disease may represent a prolongation of the fetal state(1). On 3 occasions we were able to examine specimens of human fetal liver obtained by therapeutic abortion during the fourth to fifth months of gestation. In all cases appreciable glucose-6-phosphatase activity was found. These studies have prompted an investigation of embryonic development of hepatic gluconeogenesis and hepatic glucose formation. Since the avian embryo develops as an isolated system without a constant supply of glucose from the maternal circulation, it seemed possible that glucose-6-phosphatase activity would appear early in embryonic development. In the present study the onset and extent of glucose-6-phosphatase activity during development of the chicken embryo has been investigated. The activity of β-glucuronidase was also determined since both glucose-6-phosphatase and β-glucuronidase are localized in the microsomal subcellular fraction of liver. The overall metabolic potential of embryonic liver for gluconeogenesis and glucose formation was investigated. Liver slices were incubated with pyruvate-2-C^{14} and incorporation of labeled carbon into glucose, glycogen, fatty acids and CO_2 was determined.

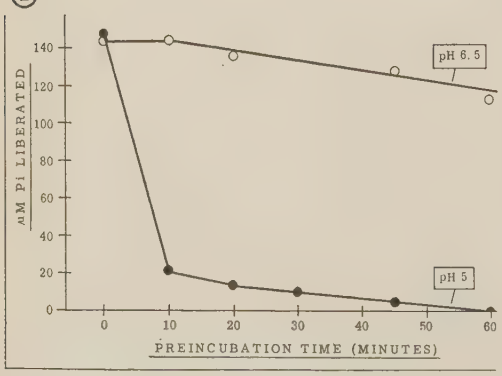
Materials and methods. Chicken embryos of one to 7 days of development were rapidly separated from the yolk sac and chorioallantoic membrane and homogenized in water. After the seventh day of incubation the liver was large enough to be quantitatively removed from the embryo. Livers were frozen in a dry ice bath, weighed and homogenized with water, 1 ml/100 mg of tissue. Microsomes were prepared from pooled samples of livers from several dozen 13- to 15-day chicken embryos, homogenized in cold 0.25 *M* sucrose, and the microsomal fraction collected in the Spinco Ultracentrifuge (Model L) according to the method of Hogeboom *et al.*(4). The microsomal fraction was resuspended in isotonic KCl to make a final volume equivalent to 1.0 to 1.5 ml/g of liver. *Enzyme assays.* Glucose-6-phosphatase activity was assayed by release of inorganic phosphate from glucose-6-phosphate incubated in citrate buffer, pH 6.5 as previously described(5). Glucuronidase activity was determined using phenolphthalein glucuronide as a substrate and acetate buffer, pH 5.0 according to the method of Fishman and Bernfeld(6). *Liver incubations.* Approximately 500 mg (wet wt.) of tissue slices were incubated with 5 ml of Hastings medium(7) containing pyruvate-2-C^{14} (40 mM) as added substrate. All flasks were gassed for 5 minutes with 95% oxygen: 5% carbon dioxide and incubated for 90 minutes at 38°. The incubation medium was analyzed initially and finally for glucose(8) and pyruvate(9) and the tissue slices analyzed for glycogen(10) and fatty acids(11). For radioassay pyruvate was plated and counted as the dinitrophenylhydrazone, glucose as phenylglucosazone, CO_2 as barium



1



2



3

FIG. 1. Glucose-6-phosphatase and β-glucuronidase activity in developing chicken embryos. Curve A, glucose-6-phosphatase; 1 unit equals amount of enzyme which will split 1 μM of substrate in 20 min. Curve B, liver wt. Curve C, glucuronidase: 1 unit equals amount of enzyme that splits 0.1 μM of substrate in 60 min.

carbonate and fatty acids plated directly on lens paper(11).

Results. Glucose-6-phosphatase activity is present in chicken embryo of 60 or more hours of age (Fig. 1). Since development of the liver begins around the 48th hour it is presumed that the appearance of glucose-6-phosphatase activity is more or less coincident with development of liver cells. That the activity observed in the early embryos was localized in the liver is suggested by the observation that no appreciable glucose-6-phosphatase activity was found in 7-day embryos from which the liver was removed.

Liver glycogen and fat content as well as β-glucuronidase and glucose-6-phosphatase activities of embryonic and adult livers are compared in Table I. Glycogen content of the liver increases during 6th to 21st days of incubation, but markedly decreases during hatching. Fat content of the liver appears to remain relatively constant during the process of embryonic development. Glucose-6-phosphatase and β-glucuronidase activities per g wet liver remain relatively constant, although total activity of both enzymes is increased in proportion to the increase in liver weight. That 2 microsomal enzymes of presumably quite different function increase in direct proportion to liver mass suggests that the hepatic cells formed may be fully functional.

Properties of microsomal glucose-6-phosphatase. To determine whether the microsomal glucose-6-phosphatase activity from embryonic chicken liver was similar in properties to enzyme found in human and rat liver, pH optimum, substrate specificity and inactivity at pH 5 were studied. The pH activity curve (Fig. 2) and substrate specificity (Table II) of chick embryo glucose-6-phosphatase was found to be the same as that observed in human and rat(12) liver.

FIG. 2. pH activity curve—Glucose-6-phosphatase activity of embryonic liver of microsomal fraction expressed as μM of inorganic phosphate (P_i) liberated during a 20 min. assay is plotted as a function of pH.

FIG. 3. pH inactivation of glucose-6-phosphatase from chicken embryo liver microsomes. Activity, expressed as μM of inorganic phosphate (P_i) liberated in a standard assay (pH 6.5) has been plotted as a function of time after preincubation of homogenates at pH 6.5 and 5.0.

TABLE I. Glycogen, Fatty Acid, Glucose-6-Phosphatase and β-Glucuronidase Content of Embryo and Adult Chicken Liver.

No. obs.	Age, days	Embryo wt, g	Liver wt, mg	L/E × 100	Liver: Glycogen, μmoles/g	Liver: Fatty acids, mg/g	Liver: Glucuronidase, mg/g	Liver: G-6-Pase, μmoles P_i/g
4	6- 7	.8 ±0	8 ± 1	1.09 ±.15	5.2 ± 2.7	61.2 ±11.4		
4	8- 9	2.1 ± .1	31 ± 3	1.49 ±.12	18.7 ± 5.6	27.7 ± 5.8		
4	9-10	2.9 ± .1	35 ± 2	1.19 ±.06	51.3 ± 4.0	31.4 ± 7.0		
3	12-13	6.8 ± .1	125 ± 9	1.84 ±.10	22.0 ± 6.3	47.0 ± 4.7	3.5 ± .3	365 ± 2
5	13-14	9.5 ± .5	170 ± 9	1.80 ±.07	73.0 ±10.3	39.4 ± 1.5	4.3 ± .7	355 ± 15
4	14-15	12.6 ± .9	238 ±28	1.88 ±.11	81.2 ±13.3	36.6 ± 2.1	4.4 ± .1	323 ± 16
4	15-16	14.8 ±1.1	293 ±23	1.98 ±.07	160.0 ± 7	41.2 ± 2.9	4.7 ± .8	286 ± 10
5	16-17	20.3 ±1.4	402 ±27	1.98 ±.04	158.0 ±20	35.7 ± 1.5	3.4 ± .7	300 ± 12
2	21-22	43.7 ±2.9*	635 ± 7*	1.46 ±.11*	46.5 ± 5.5*	32.2 ± 1.6*	4.5 ± .2*	307 ± 51*
4	Adult	808 ± 39	16800 ± 500	2.08 ±.07	141.0 ± 16	33.0 ± 1.8		251 ± 15

* Spread instead of stand. error.

Since glucose-6-phosphatase activity of rat liver microsomes is rapidly destroyed below pH 5.5(13), this process was used to separate activity of glucose-6-phosphatase from that of acid phosphatase. When embryonic chicken liver microsomes are preincubated at pH 5 there is a rapid loss of phosphatase activity (Fig. 3), thus eliminating the possible contribution of acid phosphatase to activity measured at pH 6.5.

TABLE II. Relative Rates of Hydrolysis of Various Substrates by Liver Microsomes. Conditions used for hydrolysis of all substrates were the same as those described for glucose-6-phosphate in text. Rates of hydrolysis of various substrates are expressed in relation to glucose-6-phosphate hydrolysis, arbitrarily expressed as 100.

Substrate	Rate
Glucose-6-phosphate	100
Galactose-6- "	28
Glucose-1- "	5
β-Glycerol "	4

Utilization of C^{14} pyruvate. Pyruvate is readily utilized by embryo liver (Table III) and labeled carbon from this substrate is incorporated into glycogen, glucose, fatty acids and CO_2. These data demonstrate the ability of embryo liver to form glucose both by glycogenolysis and gluconeogenesis. The appearance of radioactivity in CO_2 suggests the presence of an active citric acid cycle and respiratory enzymes. The slight incorporation of C^{14} into fatty acids is not too surprising when one considers that the yolk which serves as the energy store for the embryo is primarily lipid.

These studies suggest that early in development the chicken embryo liver is functionally complete with respect to the various parameters measured and probably is capable of releasing glucose with the appearance of the first hepatic cells.

Summary. Hepatic glucose-6-phosphatase activity appears in developing chicken embryo almost simultaneously with appearance of first hepatic cells. Glucose-6-phosphatase and β-glucuronidase activity of embryo increase in direct proportion to liver weight. Properties of embryonic liver glucose-6-phosphatase are similar to those found in adult liver of other species. Studies with liver slices indicate that the embryo liver is capable of glycogenolysis and glucose formation.

TABLE III. Incorporation of Carbon from Pyruvate into Glycogen, Glucose, Fatty Acids and CO_2 by Liver Slices from Embryonic Chickens.

Age of embryo, days	No. obs.	Pyruvate utilized	Glycogen: Net change	Glycogen: Pyruvate to	Glucose: Net change	Glucose: Pyruvate to	Pyruvate to: F.A.	Pyruvate to: CO_2
13	4	111 ± 7	−14.1 ± .5	.9 ± .1	26 ± 2	18 ± 1	.2	18.3 ± .6
15	8	96 ± 9	−55 ±4	.4	54 ± 6	34 ± 3	.2	14 ± .2*

All values as μmoles/g wet liver/90 min. Values with ± sign are mean and S.E.

* Avg of 4 determinations.

1. Nemeth, A. M., *J. Biol. Chem.,* 1954, v208, 773.
2. Weber, G., Cantero, A., *Cancer Research,* 1955, v15, 679.
3. Cori, G. T., Cori, C. F., *J. Biol. Chem.,* 1952, v199, 661.
4. Hogeboom, G. H., Schneider, W. C., Palade, G. E., *ibid.,* 1948, v172, 619.
5. Ashmore, J., Hastings, A. B., Nesbett, F. B., *Proc. Nat. Acad. Sci. U. S.,* 1954, v40, 673.
6. Fishman, W., Bernfeld, P., in Colowick, S. P., and Kaplan N. D., *Methods in Enzymology,* vI, 262 Academic Press (1955).
7. Hastings, A. B., Teng, C. T., Nesbett, F. B., Sinex, F. M., *J. Biol. Chem.,* 1952, v194, 69.
8. Nelson, N., *ibid.,* 1944, v153, 375.
9. Friedemann, T. E., Haugen, G. E., *ibid.,* 1943, v147, 415.
10. Good, C. A., Kramer, H., Somogyi, M., *ibid.,* 1933, v100, 485.
11. Renold, A. E., Hastings, A. B., Nesbett, F. B., Ashmore, J., *ibid.,* 1955, v213, 135.
12. Ashmore, J., Weber, G., *Vitamins and Hormones,* 1959, v27, 91.
13. de Duve, C., Beaufay, H., *Bull. soc. chim. biol.,* 1951, v33, 421.

Received May 27, 1960. P.S.E.B.M., 1960, v104.

Teratogenic Action of a Hypocaloric Diet and Small Doses of Cortisone.* (25895)

H. KALTER (Introduced by Roger C. Crafts)
Children's Hospital Research Fn. and Dept. of Pediatrics, College of Medicine, University of Cincinnati, Ohio

Cortisone acetate produced cleft palate in offspring of treated pregnant mice(1), in various frequencies depending on genetic and certain non-genetic variables involved(2,3,4). Supplemental treatment with riboflavin, folic acid, protein, or carbohydrate did not influence the incidence of the defect(5). The following study was undertaken to investigate the effect on fetal development of restricted daily food intake and smaller doses of cortisone than have usually been used in the past.

Materials and methods. Mice were young adult B6AF1 females, from the cross C57BL/6J ♀ x A/J ♂. They were bred to A/J males and put into individual cages upon observation of a vaginal plug, a sign to indicate that ⅓ day had passed since conception. Females were weighed at this time and divided into 5 groups of approximately similar weight composition. The groups were treated as follows: Group 1, 0.5 mg, Group 2, 1 mg, cortisone acetate/day for 4 consecutive days, beginning 11⅓ days after conception (called 0.5 and 1 mg cortisone, respectively). Group 3, given a pellet of 2.5 g food/day for 5 consecutive days, beginning 10⅓ days after conception (called restricted diet). Group 4, 0.5 mg cortisone + restricted diet. Group 5, 1 mg cortisone + restricted diet. Cortisone (Merck) was administered intramuscularly in the thigh. The food, Purina Lab Chow, was fed *ad lib.* at all times, except to females when on restricted diet. Fresh tap water was available always. Females were killed at 17⅓ days after conception, *i.e.,* about 2 days prepartum, resorption sites counted, and young removed, dried, weighed to nearest hundredth of g, and examined.

Results. Fig. 1 shows mean weights of 5 groups of females during pregnancy. Females treated only with cortisone continued gaining weight throughout treatment period, but mice fed the restricted diet lost weight early in treatment period, then became stabilized in weight, until *ad lib.* feeding was resumed, and animals given both treatments concurrently lost more weight and for a longer period than those of Group 3. The Fig. also shows that females given the restricted diet attained at time of sacrifice about the same weight as those given cortisone alone, but that animals given combined treatments did not catch up (mean weight in g, at 17⅓ days after concep-

* Supported in part by grants from Nat. Inst. Health, U.S.P.H.S. (RG-7107) and Nat. Vit. Fn.,N.Y.

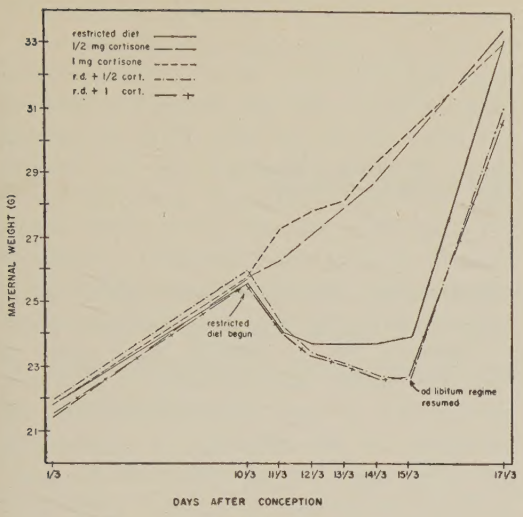


FIG. 1.

tion, for groups 1-3 = 33.2 ± .5; for groups 4 and 5 = 31.1 ± .6; $P<.01$).

Table I contains various relevant data. In view of the inverse association (3,4,6) between frequency of cortisone-induced cleft palate and maternal weight at conception, and the results to be presented, it should be noted that mean maternal weights at 1/3 day after conception of the 5 groups were greatly alike and that decrease in mean litter size was roughly proportionate to increase in resorption rate.

Number and frequency of abnormal young are found in Table II. It is seen here that 0.5 mg cortisone produced a minute incidence of young with cleft palate, namely 1.4%, whereas 1.0 mg cortisone produced 12% cleft palate. A quite unexpected result (Table II) is that 5.6% of young had cleft palate after their mothers were fed the restricted diet during pregnancy. Further, this Table shows that the restricted diet plus either dose of cortisone greatly increased frequency of cleft palate over that produced by that dose of cortisone or by the restricted diet, alone, to 36.8 and 50.7% respectively.

It is also seen from Table II that percentage of litters containing abnormal young increased with greater incidence of cleft palate, indicating that the increased rate of the anomaly was not due to greater numbers of abnormal offspring per defective litter, but that the malformation occurred in more litters. Finally, Table II shows that there occurred for most groups a decrease in mean fetal weight with increasing cleft palate frequency.

Discussion. Several points of interest have emerged from this experiment. First, it was found that simple underfeeding of a whole diet at a certain time in pregnancy is in itself teratogenic. A past study (7) showed that administration of 2.5 mg of cortisone/day for 4 consecutive days (11 1/3-14 1/3 days after conception) to pregnant mice caused food con-

TABLE II. Fetal Weight and Incidence of Cleft Palate (CP).

Treatment*	Fraction with CP	% CP	% abnormal litters	Fetal wt (g)†
.5 mg cortisone	2/144‡	1.4	10.5	.86 ±.01
1.0 " "	17/142	12.0	35.0	.74 "
Restricted diet (R.D.)	9/161	5.6	21.7	.72 "
R.D. + .5 mg cort.	42/114	36.8	82.4	.67 "
R.D. + 1.0 mg cort.	69/136‡	50.7	90.9	.67 "

* See *Materials and methods* for details.
† Mean ± stand. error.
‡ Less 2 and 1 young, respectively, with cleft lip and palate, a condition that occurs spontaneously in a low incidence in mice of this genetic composition.

TABLE I. Maternal Weight, Litter Size, and Percent Resorbed.

	Females			Offspring		
Treatment†	No.	Wt* (g) at Conception	Wt* (g) at Sacrifice	No.	Litter size*	% resorbed
.5 mg cortisone	19	21.4 ± .5	33.4 ± 1.2	146	7.7 ± .4	4.6
1.0 " "	20	21.8 ± .4	33.1 ± .8	142	7.1 ± .4	4.7
Restricted diet (R.D.)	23	21.8 ± .5	33.2 ± .6	161	7.0 ± .3	8.0
R.D. + .5 mg cort.	17	21.9 ± .7	31.1 ± 1.0	114	6.7 ± .5	16.8
" + 1.0 " "	22	21.6 ± .5	31.0 ± .9	137	6.2 ± .4	18.4

* Mean ± stand. error.
† See *Materials and methods* for details.

sumption to be elevated from the control mean for these 4 days of 5.8 g food/day to 6.6 g/day for the same 4 days, an increase of 14%. The amount fed in this study, 2.5 g/day, is 43% of control value, and 38% of experimental value. It can be taken, therefore, that the mice fed the restricted diet in the present study consumed about 40% of an *ad lib.* intake.

Starvation for 24 hours on 9th day of pregnancy was found to produce exencephaly and costal and vertebral defects in the 129 strain of mice(8), and for 72 hours from 9th to 12th days of pregnancy to produce cleft palate in mice of a different genetic constitution(9). But these results were not preparation for the finding that a hypocaloric diet would be teratogenic.

The second point of interest is that 1.0 mg cortisone produced so much greater an incidence of cleft palate than 0.5 mg cortisone (1.39% *vs.* 11.97%; $\chi^2 = 11.3$, $P<.001$). Apparently a threshold of sensitivity is crossed in this experimental setup by a dose lying somewhere between these amounts of the hormone.

Third, it is of great interest that a synergistic effect resulted from simultaneous treatment with the restricted diet and doses of cortisone. In Table II, however, it can be noted that the lesser amount of cortisone plus restricted diet had a larger relative effect than the greater dose of cortisone plus restricted diet. In the first case the combined treatment resulted in a percentage of cleft palate 5.3 times that of the sum of the individual treatments whereas with the second combination this was 2.9 times the sum of the treatments alone. It seems therefore that the smaller dose of cortisone, although producing by itself a negligible incidence of the defect, sensitizes the maternal-fetal system to a relatively greater extent to a concomitant embryopathogenic circumstance than does the larger dose.

Summary. Two relatively small doses of cortisone acetate administered to pregnant mice caused 1.4 and 12.0% cleft palate, respectively, in the offspring. A hypocaloric diet, consisting of about 40% of *ad lib.* daily intake, given during middle 3rd of pregnancy, produced 5.6% cleft palate. The doses of cortisone plus the restricted diet caused 36.8 and 50.7% cleft palate, respectively. These results are discussed.

1. Fraser, F. C., Fainstat, T. D., *Pediatrics,* 1951, v8, 527.
2. Kalter, H., *Genetics,* 1954, v39, 185.
3. ———, *Am. J. Physiol.,* 1956, v185, 65.
4. ———, *J. Exp. Zool.,* 1957, v134, 449.
5. ———, *Plastic Reconstr. Surg.,* 1959, v24, 498.
6. ———, *Genetics,* 1957, v42, 380.
7. ———, *Canad. J. Biochem. Physiol.,* 1955, v33, 767.
8. Runner, M. N., Miller, J. R., *Anat. Rec.,* 1956, v124, 437.
9. Kalter, H., *Genetics,* 1954, v39, 975.

Received June 10, 1960. P.S.E.B.M., 1960, v104.

Friends of the Society

Our members are requested to note the following list of Friends. We wish to express our thanks to each of them. Their contributions help materially in meeting the very sharp increase in costs of publication.

FRIENDS

Abbott Laboratories, Research Division
American Cancer Society
American Cyanamid Co.
American Tobacco Company
Ayerst Laboratories
Bristol Laboratories, Inc.
Burroughs Wellcome Co., Inc.
Carter Products, Inc.
Ciba Pharmaceutical Products, Inc.
Eaton Laboratories, Division of the Norwich Pharmacal Co.
Louis and Bert Freedman Foundation
Geigy Pharmaceuticals, Division of Geigy Chemical Corporation
Hoffmann-LaRoche, Inc.
Eli Lilly and Co.
Lipotropic Research Foundation
Mead Johnson & Co.
Merck Institute for Therapeutic Research
The Wm. S. Merrell Co.
Miles Laboratories, Inc.
National Drug Company
Ortho Pharmaceutical Corporation
Parke Davis and Co.
Chas. Pfizer and Co., Inc.
Pitman Moore Co.
Riker Laboratories, Inc.
The Rogoff Foundation
Damon Runyon Memorial Fund
Rutgers Research & Endowment Foundation
Sandoz Pharmaceuticals
Schering Corporation
G. D. Searle and Co.
Sharp and Dohme, Division of Merck & Co., Inc.
Smith Kline and French Laboratories
Carroll Dunham Smith Pharmacal Company
Squibb Institute for Medical Research
Sterling-Winthrop Research Institute
G. N. Stewart Memorial Fund
U.S. Vitamin Corporation
The Upjohn Co.
Warner-Chilcott Laboratories
The Wilson Laboratories
Wyeth Laboratories
Anonymous

CONTRIBUTION received from Johnson and Johnson